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        Quantitative PCR

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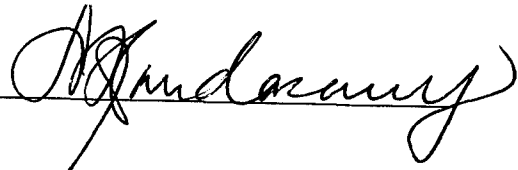
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GRANT REPORT
Indra Poola, Ph.D

1. INTRODUCTION

Breast cancer is a tumor of the mammary gland epithelium affecting about 176,000 women in the western hemisphere and killing about 50,000 people every year in the United States. A substantial body of epidemiological, experimental, and clinical evidence indicates that exposure to the natural hormones, estrogen, progesterone and prolactin, which are important for the normal development and function of the breast tissue, play a major role in the growth of breast cancer cells and may even contribute to the development of breast cancer.

A. Prognostic factors in breast cancer. Once diagnosed with breast cancer, therapeutic approaches, survival rate and disease recurrence depend upon the expression of the prognostic factors, hormone receptors, growth factor receptor superfamily and proteins involved in invasive metastatic phenotype in the tumor tissues.

B. Hormone receptors in the prognosis and therapy of breast cancers. The most important among prognostic factors are the hormone receptor, estrogen-, and progesterone receptors (ER and PgR). <u>Estrogen receptor</u>. The presence of ER in tumors indicates a good prognosis and the patients respond to anti-estrogen therapies. The expression of various molecular forms of ER was studied in several breast cancer cell lines and tissues. Analysis of the estrogen receptor mRNA has shown that it undergoes alternate / inaccurate splicing in the tumor tissues giving rise to several aberrant (variant) forms of the receptor molecules. These studies raise the possibilities of various species of ER which have exon deletions/truncations in the 1) estrogen binding region (exons 4-8), 2) DNA binding region (exons 2-3) and 3) other portions of the receptor molecule.

C. Current methods of estrogen receptor evaluation and their disadvantages.. The presence of estrogen receptor is the basis for anti-estrogen therapies to treat breast cancer and its presence also indicates good prognosis, longer survival and low recurrence rates. Because of these therapeutic and prognostic reasons, estrogen receptor is detected and quantitated in tumors. Currently its detection and quantification are done using immunohistochemistry procedures in clinical laboratories.While these provide information on the presence of ER, they are 1) very cumbersome, 2) time consuming, 3) not highly sensitive, 4) require a large sample 5) **do not yield quantitative information**, 6) not suitable to evaluate in fine needle aspirations 7) very expensive to the patient and 8) **Most importantly, immunohistochemical assays cannot distinguish between the wild type and variant forms of ER, therefore, cannot predict hormone therapy responders precisely. Because of the therapeutic considerations, there is an urgent need to rapidly quantitate and get a profile of wt and vt of ER to predict hormone therapy**

responders and disease prognosis.. Furthermore, due to increased awareness and improved methods of detection, smaller and smaller tumors are detected in recent times which restricts the amount of tumor tissue available for the immunohistochemisty. This led to a proposal that molecular biological methods may be preferred since they require very small samples. In addition, several prognostic factors could be detected at the same time by these procedures.

The essence of our proposal is to develop highly sensitive cost effective quantitative PCR assays to measure a multitude of hormone receptors and other diagnostic and prognostic factors in breast cancers.

D. Development of new prognostic assays for estrogen receptor. In our grant, we proposed to develop highly sensitive, rapid, cost-effective PCR based methods to quantitate ER and other prognostic factors in a small amount of clinical samples. The developed assays could be used virtually in every clinical lab to diagnose and evaluate prognosis of breast cancers. The significance of this assay is that patients could then be identified who are most likely to respond to hormone therapy and that the overall prognosis may be assessed.

2. SPECIFIC AIMS.

The specific aims of our proposal are to develop highly sensitive PCR methods for rapid, specific, diagnosis of breast cancers on the basis of their estrogen- and progesterone receptor expression.

3. BODY OF THE REPORT (STUDIES AND RESULTS).

We have developed the state-of-the art molecular methods for the detection and quantification of estrogen receptor in breast tumors. In particular we have developed molecular procedures to

1) detect and quantify wild type estrogen receptor alpha mRNA, 2) analyzed the alterations in the estrogen receptor mRNA in the breast tumors of Caucasian and African American women 3) devised novel "Splice Targeted Primer Approach" for detecting the estrogen receptor alpha mRNAs which have deletions in one or more exons 4) Identified twenty alternatively spliced ER alpha mRNAs which have deletions in various combinations of exons in breast cancer cell lines and tumors and 5) developing methods for the quantification of estrogen receptor exon-deletion variant mRNAs in breast cancer cell lines and tumors. These accomplishments are described briefly here.

A. Development of molecular methods to quantitate estrogen receptor mRNA copy numbers. We have devised a method to quantify the mRNA copy numbers of estrogen receptor by reverse transcription polymerase chain reaction (RT PCR) template competition method. We were the first to develop the quantitative molecular method for the quantification of the receptor mRNA in tumor samples. The procedure is described in detail in the accompanying publication.

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Our work on the quantitation of estrogen receptor copy numbers is published in Analytical Biochemistry. A copy of the paper is attached.

B. Studying the estrogen receptor profiles in the breast tumors of Caucasian and African American women. After developing the quantification methods for the ER wild type mRNA, we wanted to apply these methods for the quantification of ER in tumor samples. Therefore, we started collecting the tumor samples from Howard university hospital where a large number of breast tumor biopsies are performed. Since the majority of biopsies performed at Howard University hospital are on African American women, it gave us an opportunity to look at any alterations in the ER in these samples. Several reports indicate that the incidence of breast cancer in young African American women is higher and lower in older women compared to Caucasian women. However, the mortality rate with breast cancer is three times as high in African American women compared to women in other populations. The high mortality rate does not appear to be due to differences in socioeconomic status, stage of diagnosis, age, known risk factors or treatment. It has been hypothesized that breast tumors across racial and ethnic groups could have different biologic characters which may account for survival disparities. However, such factors are not identified thus far. There also appears to be differences in tumor biology. The breast tumors from African American women in general are poorly differentiated with lower frequency of the steroid hormone receptors, the estrogen- and progesterone receptor, which are generally associated with well or moderately differentiated tumors and their presence indicate good prognosis. It is not known whether this aggressive tumor phenotype contribute to high mortality with breast cancer. All these studies indicate that the mortality rate is related to biological factors. Preventive and therapeutic strategies can be devised if those factors are identified in the tumors of African American women. Retrospective studies conducted on tumor tissues could be highly valuable in identifying those factors.

To understand the molecular factors which may be responsible for the poorly differentiated aggressive tumors in African American women, we studied the most important prognostic factor, the estrogen receptor in the tumor tissues. In particular, we investigated the alteration in the expression of estrogen receptor gene in the freshly collected breast tumors. Briefly, we studied the estrogen receptor mRNA in 25 immunohistochemically estrogen receptor positive and 5 immunohistochemically negative tumors from African American women and 20 ER-positive tumors from Caucasian patient tumors (obtained from Dr. Suzanne Fuqua at San Antonio, TX). We studied the receptor transcripts by reverse transcription polymerase chain reaction (RT PCR) using a number of primer pairs. Our results indicated that only a third of the tumors have full length receptor transcripts and the majority of tumors had at least three types of modifications. They are 1) truncations in exon 8, which encodes for part of the hormone binding region, 2) base insertions and 3) absence of naturally occurring exon-7 deletion variant. The significance of these finding and their role in the aggressive tumor types are not clear at this point.

4

This work has been published in the Journal of Cancer Research and Clinical Oncology. A copy of the publication enclosed.

C. A novel 'Splice Targeted Primer Design' for the targeted amplification of alternatively spliced ER mRNAs. Estrogen receptor occurs both as unmodified (wild type) and several spliced variants (isoforms) which have the deletions in one or more exons. Several studies have shown that the estrogen receptor isoforms play important functional roles in the estrogen induced signal transduction processes under normal physiological conditions. Recent reports also indicate that the isoforms of estrogen receptor pattern and levels are altered in breast tumors and their content influence the response to anti-estrogen therapies. Because of the importance of isoforms, it is important to develop methods which can precisely detect and quantify their amounts. The currently available methods can only detect the most abundant forms and cannot detect the mRNAs of the low abundance. Therefore, we have developed new strategies to detect even the least abundance isoform by PCR using targeted primers. In particular we designed, tested and identified targeted primers for exon-7 deletion variant, exon-5 deletion variant, exon 2-deletion variant and exons 2-3 deletion variants.

These results are published in Analytical Biochemistry. A copy of the reprint is enclosed.

D. Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using "Splice Targeted primers". After developing the new primer design strategies for specific amplification of the mRNAs of isoforms, we used those targeted primers to analyze the variant patterns in breast cancer cell lines and tumor samples. We employed seven breast cancer cell lines, MCF-7, T47D, ZR-75,LCC1, LCC2, LCC9 and MDA-MB-435 and three tumors to test the applicability of the targeted primers. Our results showed that the targeted primers not only amplified the single deletion transcripts, but also multiple deletion isoforms. These transcripts were not detected and characterized until now because of the unavailability of the sensitive methodologies until now. The different cell lines did not differ in the single deletion transcripts. However, they differed in the presence and content of multiple deletion transcripts. Interestingly, we observed only the multiple deletion variant transcripts in the tumor samples. The twenty spliced mRNAs identified were *2Δ; 2Δ & 7Δ; 2Δ, 5Δ & 7Δ; 2Δ & 4-5Δ; 2Δ & 4-6Δ; 3Δ; 3Δ & 7Δ; 2-3Δ; 2-3Δ & 7Δ; 2-3Δ, 5Δ & 7Δ; 4Δ; 4Δ & 7Δ; 5Δ; 5Δ & 2Δ; 5Δ & 2-3Δ; 6Δ; 7Δ; 7Δ & 3-4Δ; and 7Δ & 3-5Δ* (*Δ*, deletion). Our results also suggested that the alternative splicing takes place in a sequential manner instead of at random. Deletion of an exon in the transcript, the second exon to be deleted appears to be exon 7, which is followed by exon 5. Because of this trend, the most frequently deleted exon is the exon 7 and the second most frequently deleted exon is exon 5. The estrogen dependence appear to be associated with the presence of multiple deletion (exon 3-7 and exon 4-7).

The above work has been published in the Journal of Steroid Biochemistry and Molecular Biology. A copy of the reprint is enclosed.

E. Quantification of estrogen receptor isoform mRNAs. After establishing the applicability of variant targeted primers for specific amplification of variant transcripts, we decided to apply them in quantification of their transcripts by template competitive PCR similar to the quantification of wild type estrogen receptor transcripts.

A manuscript has been published in the journal Steroids and a copy enclosed.

F. Studying the alternatively spliced ER isoforms in normal and malignant breast tissues. By employing the newly developed splice Targeted primer approach, we studied the alternatively spliced ER alpha mRNAs in normal and malignant breast tissues. Our results indicate that the alternative splicing of ER alpha mRNA is increased in tumor tissues compared to normal tissues and each normal/cancer tissue has a distinct profile of ER isoforms. The heterogeneity of ER isoforms may have implications in developing breast cancer, response to a particular anti-estrogen therapy and overall survival.

A manuscript has been communicated on this work and a copy enclosed.

4. KEY RESEARCH ACCOMPLISHMENTS.

- **Identified the alterations in the estrogen receptor mRNAs in the breast tumors of Caucasian and African American women**

- **Designed methodologies to detect and quantify ER mRNAs from a small amount of tumor tissue.**

- **Devised methods to specifically amplify the alternatively spliced estrogen receptor transcripts**

- **Identified twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors**

- **Developed methods to quantify alternatively spliced ER alpha mRNAs as separate gene populations.**

- **Identified the changes in alternatively spliced ER alpha mRNAs in cancer tissues compared to normal breast tissues.**

5. REPORTABLE OUTCOMES.

A. Publications and presentations:

1   Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using spliced targeted primer approach.
I. Poola, S. Koduri, S. Chatra and R. Clarke (2000) **J. Steroid Biochem. Mol. Biol** , 72, 249-258

2. Primer design strategies for the amplification of alternatively spliced estrogen receptors.
    D. M.Williams, S. Koduri, Z. Li, W. D Hankins, and **I. Poola** (1999)
    Analytical Biochemistry 271, 194-197.

3. Quantitation of estrogen receptor mRNA copy numbers in breast cancer cell lines and tumors.
    I. Poola, D.M. Williams, S. Koduri, J. Ramprakash, R. E. Taylor and W.D. Hankins (1998)
    Analytical Biochemistry, 258, 209-215

4. Quantitation of alternatively spliced estrogen receptor alpha mRNAs as separate gene populations in breast cancer cell lines and tumors.
    S. Koduri and **I. Poola** (2000) **Steroids. 66, 17-23.**

5. Alterations in the estrogen receptor mRNA in the breast tumors of African American women.
    S. Koduri, S. A.W. Fuqua, and **I. Poola** (2000) **J. Cancer Res. and Clinical Oncol** ,126, 291-297.

6. Microsatellite instability and loss of heterozygosity in chromosome 13q12-13 in ductal carcinoma *in situ* of the breast.
    Y. Huang, I. Russo, **I. Poola**, E. B. Chung,, and J. Russo (1998) Proceedings of the 89th Annual meeting of American Association for **Cancer Research** , A3651

7. Frequency of alternatively spliced estrogen receptor alpha mRNA is increased in breast cancer tissues.
    I. Poola, S. Koduri, and V. Speirs (2001) Submitted

8. Moecular assays for the detection and quantitation of various estrogen receptor isoforms.
    INVITED TO PRESENT AT ERA OF HOPE MEETING held in June 2000, Atlanta, GA

B. Patents and Licences

None

C. Degrees awarded

None

D. Development of cell lines and tissues

None

E. Informatics such as databases

None

F. Funding applied for based on work supported by this grant.

1. Changes in the alternatively spliced estrogen receptor alpha mRNAs in the breast

tumors compared to normal breast tissues.
Role. PI
Applied to Susan G. Komen breast cancer foundation Race for Cure and awarded
$141,000.

2. Clinically applicable molecular assay to profile estrogen receptors in breast tumors
Role. PI
Applied to National Cancer Institute and awarded $760,000 in 2000.

3. Correlation between the composition of various estrogen receptors and ligand binding.
Role. Collaborating Investigator.
Applied for Dr. K. Baldwin as HBCU/MI Focussed Training award and funded $149,944.

4. Estrogen receptor beta expression in the breast tumors of African American women.
Role. PI
Applied to susan G. Komen Breast Cancer Foundation, Race for the Cure for $105,000
(Pending).

G. Employment or research opportunity applied for

None

6. CONCLUSIONS. A highly sensitive molecular biological approaches have been developed to
detect and quantitate the exact copy numbers of wild type and various single and multiple exon
deletion forms of estrogen receptor mRNA in breast tumors. Once converted into a clinically
feasible assay, it could become a highly valuable method to precisely predict the prognosis of the
disease and identify patients who are most likely to respond to anti-hormone therapy.

7. APPENDICES.

Five reprints and one manuscript.

Quantitation of Estrogen Receptor mRNA Copy Numbers in Breast Cancer Cell Lines and Tumors

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Several clinical studies have suggested that the content of estrogen receptor (ER) in breast tumors influences the survival, tumor recurrence, and response to antiestrogen therapies. Therefore, the ability to precisely quantitate the ER content in tumor tissues will be of significant benefit to women with breast cancer. Although immunohistochemical and polymerase chain reaction (PCR) methods have been described for the detection and semiquantitation of ER, none of them precisely quantitate ER copy numbers in tumor samples. In the present report we describe a molecular approach to accurately quantitate ER mRNA copy numbers using a reverse-transcription PCR (RT-PCR) template competition method. A competitor template was devised by inserting unrelated nucleic acid sequences into an ER cDNA clone. A template competitive RT-PCR analysis was then performed to determine the number of copies of ER mRNA. As a standard of reference for the ER mRNA copy numbers from various samples, the mRNA copy numbers of a constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were also quantitated. The ER quantitations were performed in three positive cell lines, MCF-7, T47D, and ZR-75, and two positive tumor tissues by this approach. Our results described here show that among the cell lines studied, T47D expresses the highest copy numbers of ER. We also present here that ER as low as 10^3 copies per 10^5 copies of GAPDH can be detected and quantitated in tumor samples by the template competition method. In addition, the molecular approach can simultaneously detect, distinguish, and quantitate exon deletion variant copy numbers of ER. The results described in this report indicate that the ratios of exon 7 deletion variant to wild type in the tumor tissues are significantly higher than in the cell lines studied. © 1998 Academic Press

The human estrogen receptor (ER[1]) is a member of the superfamily of nuclear steroid receptors. The gene for ER is more than 140 kb in length and contains eight exons. The protein has six functional domains, designated A through F. Domain C, which spans exons 2 and 3, binds DNA and domain E, which spans exons 4–8, contains the hormone binding site (1, 2). The presence of ER in breast tumor cells is considered a good prognosis and the patients who express estrogen receptors in their tumors have an overall longer survival and lower risk of tumor recurrence (3). In addition to its prognostic value, the presence of ER is exploited to treat tumors with antiestrogen therapy (4). Clinical studies have indicated that approximately 50% of patients with ER content >1000 pM/mg protein are found to respond favorably to antiestrogen therapy. Currently, the ER status in breast tumor tissues is determined from rough estimates yielded by microscopically scoring slides subjected to immunohistochemistry techniques. In recent years, due to increased awareness and periodic screening procedures, tumors of small sizes can be detected. In these cases the limited amount of tumor tissue often restricts ER protein quantitation. Several studies have suggested that molecular biological procedures may be preferred for ER quantitation since they require much smaller amounts of tumor tissue. This led to the development of PCR-based methods for the detection and relative quantita-

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[1] Abbreviations used: ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction.

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[1] To whom correspondence should be addressed.

tion of ER mRNA in tumor samples (5–7) in recent years. However, none of the methods described thus far can estimate the absolute number of copies of the receptor mRNA.

In this report, we describe a significantly improved PCR approach with which one can quantitate the exact number of ER mRNA copy numbers of wild-type as well as variant transcripts. As a standard of reference for the ER transcripts, we also describe the quantitation of the mRNA copy number of a constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The significance of our PCR template competition assay, therefore, is twofold: (i) survival prognosis and hormone therapy response may be more readily predictable and (ii) the relationship of ER variants to breast cancer growth can be assessed.

MATERIALS AND METHODS

RNeasy midi kits, AmpliTaq PCR core kits, and QIAquick gel extraction kits were from QIAGEN Inc. (Santa Clara, CA). Reverse transcriptase kits were from Applied Biosystems. [α-^{32}P]dCTP (sp act 3000 Ci/mmol, Cat. No. AA0005) was from Amersham. A plasmid containing the full-length ER cDNA gene pIC-ER-F was obtained from ATCC. Trizol reagent for total RNA isolation was from Life Technologies. PCR quality water and Tris–EDTA buffer were from Biofluids (Rockville, MD).

Cell Lines and Breast Tumor Samples

The ER positive breast cancer cell lines MCF-7, T47D, and ZR-75, and an ER negative cell line, LCC6, were kind gifts from Dr. Robert Clark, Georgetown University School of Medicine. They were maintained in 90% IMEM without gentamycin and 10% fetal bovine serum. The breast tumor samples were derived from a breast tumor bank established by one of us (I.P.) at Howard University Medical School.

RNA Isolation and Reverse Transcription

Total RNA from breast cancer cell lines was isolated using RNeasy midi kits. This method yielded about 0.8–1.0 mg of total RNA per 10^7 tissue culture cells. Total RNA from powdered breast tumor samples was isolated using Trizol reagent. This method yielded about 30 μg of total RNA per 100 mg of tumor tissue. The isolated RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamers.

PCR

For ER cDNA amplification, the sequences between exons 4 and 8 were amplified using the sense primer 5′-GCCCGCTCATGATCAAACGC-3′ (exon 4, bp 1112–1132) and an antisense primer 5′-TACTTTTGCAAG-GAATGCGA-3′ (exon 8, bp 1978–1958) (8). The amplification was carried out in a 12.5-μl reaction volume containing the reverse-transcribed cDNA, 1× PCR buffer, 1× Q solution (Qiagen), 200 μM each of dNTPs, 2 μM each of sense and antisense primers, and 0.6 U of Taq polymerase. The PCR conditions were initial denaturation for 5 min at 95°C followed by 94°C for 1 min, annealing for 1 min at 55°C, and extension for 2 min at 72°C for 40 cycles and final extension for 10 min at 72°C. The GAPDH cDNA was amplified using a sense primer, 5′-AAGGCTGAGAACGGGAAGCTTGT-CATCAAT-3′ (exon 3, bp 241–270), and an antisense primer, 5′-TTCCCGTCTAGCTCAGGGATGACCTTG-CCC-3′ (exon 7, bp 740–711) (9, 10), under the same PCR conditions as described for ER.

Design and Construction of Competitor Template DNAs for the Quantitation of ER and GAPDH by Template Competition

In the template competition approach (11), two templates which can be amplified by the same primers are used in the PCR. If two templates are amplified at the same efficiency, the ratio of their PCR products will reflect the starting ratio of the two template DNAs. This means that if the amount of a competitor DNA is known, then the amount of unknown template can be precisely determined by measuring the two PCR products. This requires that the products generated by two different templates must be distinguishable from each other. For this purpose, a competitor plasmid DNA which contained an extra fragment of DNA internal to the PCR primer location that generates a larger PCR product was prepared.

Competitor template DNA for GAPDH. The competitor plasmid for GAPDH was constructed in two steps. First a 500-bp portion of the GAPDH cDNA was amplified by RT-PCR using RNA extracted from the breast cancer cell line MCF-7. The primers used had an attached upstream EcoRI restriction enzyme site and a downstream BamHI restriction enzyme site. The sense and antisense primers were 5′-CCCGAATTCAAGGCT-GAGAACGGGAAGCTT-3′ (exon 3, bp 241–261) and 5′-CCCGGATCCTTCCCGTCTAGCTCAGGGATG-3′ (exon 7, bp 740–720), respectively (10). This fragment was subcloned into the EcoRI and BamHI sites of pBluescript II SK+ (Stratagene). The resulting plasmid was designated as pBS-GAPDH. Next, a 210-bp fragment of DNA was amplified from bacteriophage λ DNA (from bases 1920 to 2130) (12) by PCR using primers which had attached NcoI restriction enzyme sites to both ends. The sense and antisense primers were 5′-CCCCCATGGCTGGACCGC-TACGAAATGCGC-3′ (bp 1920–1940) and 5′-CCCCCA-TGGCGTTCAACAATGGTCGGG-3′ (bp 2130–2110), re-
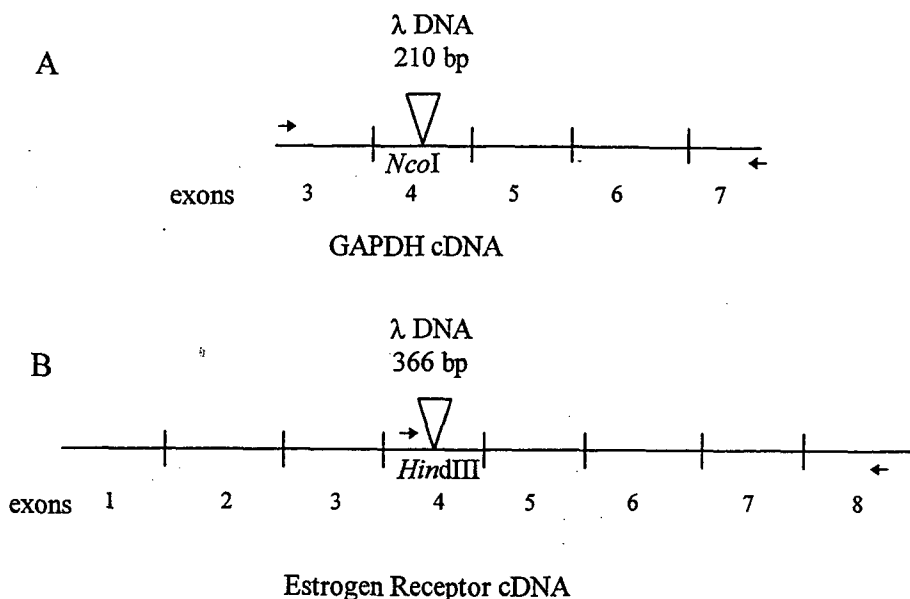
FIG. 1. Design of competitor plasmids for the quantitation of GAPDH and ER copy numbers. (A) pBS-GAPDH-λ. The approximate primer locations are indicated by arrows. A 210-bp λ DNA fragment was inserted into the NcoI site in exon 4 of the GAPDH cDNA gene. The resulting GAPDH competitor cDNA generated a 710-bp product by PCR amplification. (B) pSG-ER-λ. The approximate locations of the primers used in exons 4 and 8 are indicated with arrows. A 366-bp λ DNA fragment was inserted at the HindIII site in exon 4 of the ER cDNA. The resulting ER competitor cDNA generated a 1233-bp product by PCR amplification.

spectively. This fragment was then subcloned into the unique NcoI site within the 500-bp piece of pBS-GAPDH. The competitor thus generated was termed as pBS-GAPDH-λ. The schematic representation of the competitor design for GAPDH is shown in Fig. 1A. When pBS-GAPDH-λ was used in a PCR, it generated a fragment 210 bp larger than pBS-GAPDH (not shown).

Competitor template DNA for ER. The competitor plasmid for ER was prepared by inserting λ sequences into an unique HindIII site in exon 4 of ER cDNA. This construction was also performed in two steps. First, since the commercially procured pIC vector in which ER was cloned (13) contains an additional HindIII site, the ER cDNA gene was subcloned from pIC-ER-F into the EcoRI site of the plasmid pSG5 (Stratagene). Second, a fragment of DNA was amplified from bacteriophage λ DNA using the sense primer 5'-GGGAAGCTTAAACCATTCT-TCATAATTCAA-3' (bp 37101–37121) containing a 5' HindIII linker and the antisense primer 5'-CGCACCAA-CAGGCTCCAAGCC-3' (bp 37465–37485) which flanked a native HindIII site in λ (12). This fragment generated a 366-bp DNA when digested with HindIII. The 366-bp fragment generated above was inserted into the HindIII site in exon 4 of ER. The competitor thus generated is termed pSG-ER-λ. The schematic representation of the design of the competitor for ER is shown in Fig. 1B. As shown in the figure, the primers used for amplification of ER flank the unique HindIII site within exon 4. When this cDNA was used as a competitor template in a PCR with the ER primer pair which can amplify exons 4–8, it

generated a product that is 366 bp larger than the normal product and thus distinguishable by gel electrophoresis (data not shown).

Competition Assay

The ability of the competition assay to accurately measure ER and GAPDH transcript copy numbers was first confirmed by performing the assay with known amounts of both the competitor templates and the cloned normal GAPDH and ER cDNA genes. The numbers of copies of normal and competitor DNA molecules were determined by multiplying the average mass of a base pair (660 daltons) by the number of base pairs and then dividing Avagadro's number by that value as in the following example for a 3500-bp DNA molecule: $(6.023 \times 10^{23}$ molecules/mol)/3500 bp $\times$ 660 g/mol/bp $= 2.61 \times 10^{17}$ molecules/g $= 2.61 \times 10^{11}$ molecules/μg. For these experiments the number of copies of ER cDNA and GAPDH cDNA was kept constant at 10^7 while the concentrations of the competitor DNAs ranged from 10^4 to 10^8 copies. In both ER and GAPDH competition assays, we expect the normal and the competitor plasmid DNAs to cross over at equimolar concentrations (10^7 copies). It was found that the amounts of competitors needed to generate equal ratios of the normal and the competitor PCR products for GAPDH and ER were 10^7 and $10^{7.25}$, respectively. They crossed over as expected or within the range of 1.5–2 times (data not shown), which agrees with the previously

Number of Copies of Competitor x 10⁵

GAPDH Competitor
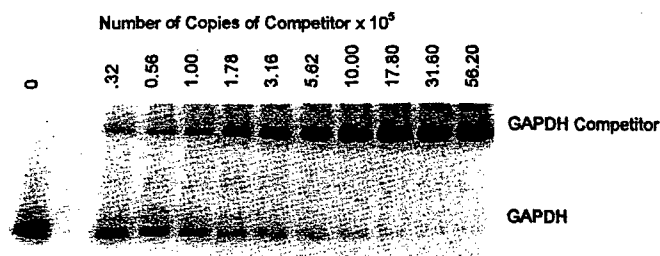
GAPDH

FIG. 2. Template competition assay for GAPDH quantitation in the T47D cell line. PCR amplification with GAPDH specific primers and a constant amount of cDNA prepared by reverse transcription of 50 ng of T47D total RNA was performed in the presence of varying amounts of the competitor pBS-GAPDH-λ. The number of copies of the competitor ranged from 0.32×10^5 (lane 2) to 56.2×10^5 (lane 11) in half-log increments. The PCR amplifications were conducted under the conditions described under Materials and Methods. The amplified products were electrophoresed in 4.5% acrylamide gels and detected by autoradiography.

published reports on the template competition method (11). After verifying the feasibility of the template competition method, we went on to measure the copy numbers of both ER and GAPDH in breast cancer cell lines and tumor samples. To determine ER copy numbers, the cDNA prepared from 250 ng of total RNA was kept constant and the competitor concentration was increased from 10^4 to 10^8 copies in quarter-log dilutions. To determine the number of copies of GAPDH, the cDNA prepared from 50 ng of total RNA was kept constant and the competitor concentration was increased from 10^4 to 10^8 in half-log dilutions. The PCRs were performed as described before.

Detection and Quantitation of PCR Products

For quantitation of the PCR products, $[\alpha\text{-}^{32}P]dCTP$ was included in the PCR at 0.5% of the total reaction volume. The radiolabeled PCR products were electrophoresed in 4.5% acrylamide gels, 90 mM Tris–borate, and 0.2 mM EDTA at a 40-mA constant current for 3.5 h. The gels were dried and autoradiographed. The radioactivity in the individual PCR products was quantitated by scanning the autoradiograms in a laser densitometer (Molecular Dynamics).

RESULTS AND DISCUSSION

Clinical studies have suggested that the content of ER in the tumor tissues influences the prognosis and response to antiestrogen therapy in breast cancer (3, 4). Therefore, the ability to precisely quantitate the ER content in the tumor tissues will be of significant benefit to women with breast cancer. Although immunohistochemical and several PCR-based methods have been described, none of them quantify the exact copy numbers of ER in tumor tissues. In the present study we describe a molecular approach to precisely quantitate the ER mRNA copy numbers in the tumor tissues by the template competition method. We have also devised a method to quantitate the exact mRNA copy numbers of a constitutively expressed gene, GAPDH, so that the ER copies from various samples can be expressed with reference to the copy numbers of GAPDH. In addition, we show that the template competition method described here can simultaneously quantitate the copy numbers of ER exon Δ variants. The presence of the variant ERs which have deletions
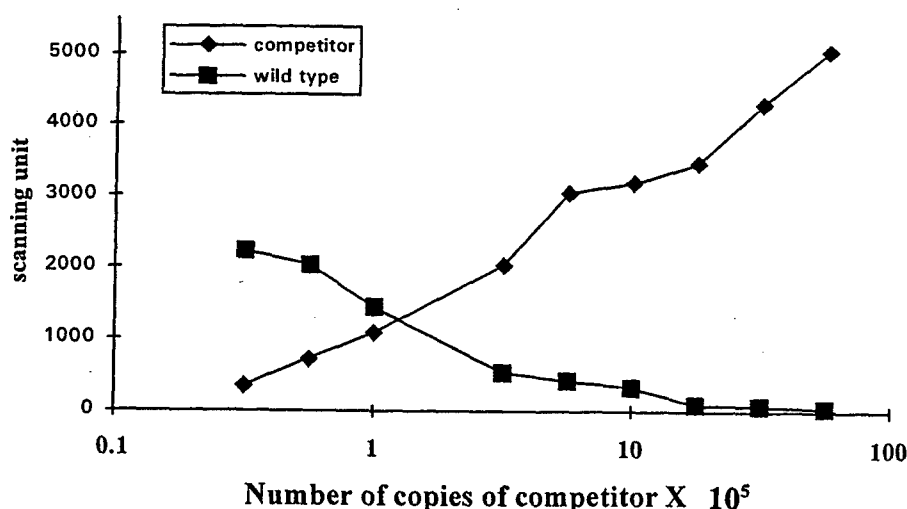
FIG. 3. Absolute quantitation of GAPDH copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The normalized scanned units were plotted against the number of copies of the competitor pBS-GAPDH-λ in the PCR. The cross-over point is shown at which the GAPDH products generated from both the competitor and T47D cDNA are equal. The number of copies of GAPDH transcripts is equal to the number of copies of the competitor at the cross-over point.
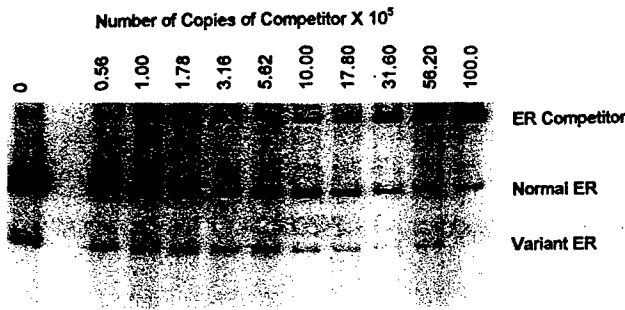
FIG. 4. Template competition assay for ER quantitation in the T47D cell line. PCR amplification with ER specific primers and a constant amount of cDNA prepared by reverse transcription of 250 ng of T47D total RNA was performed in the presence of varying amounts of the competitor pSG-ER-λ. The number of copies of the competitor ranged from 0.58 × 10^{5} (lane 2) to 100 × 10^{5} (lane 11) in quarter-log increments. PCR amplifications were conducted under the conditions described under Materials and Methods. The amplified products were electrophoresed in 4.5% acrylamide gels and detected by autoradiography. Two ER products from cDNA derived from the T47D cell line corresponding to wild-type and exon 7Δ variant are seen and designated as normal ER and variant ER.

in exons 2, 3, 2–3, 5, and 7 in both normal mammary and breast tumor tissues is well documented (14–16).

We designed and cloned the competitor templates which enabled us to quantitate the exact copy number of both GAPDH and ER transcripts by template competition. For ER quantitations, we used the primers which amplified sequences between exons 4 and 8. The primers were first tested on the cDNA prepared by reverse transcription of total RNA from various breast cancer cell lines. We observed the expected 865-bp PCR product plus an additional product size of about 700 bp in MCF-7, T47D, and ZR-75 and the two tumors tested. Neither of these products was observed in cell line LCC6 which is typed as ER negative. Restriction enzyme digestion of the 700-bp fragment suggested that it was the previously characterized exon 7Δ variant and subsequent sequence analysis confirmed its identity (data not shown). After verifying the identity of the 700-bp fragment, we performed the quantitations of GAPDH and ER copy numbers in the ER positive breast cancer cell lines and tumor tissues.

A typical quantitation assay performed for GAPDH using the reverse-transcribed cDNA prepared from the T47D cell line is presented in Figs. 2 and 3. The amount of competitor in the PCR ranged from 0.32 × 10^{5} copies (Fig. 2, lane 2) to 56.2 × 10^{5} copies (Fig. 2, lane 11). As seen in Fig. 2, the GAPDH PCR product from the T47D cell line (lower band) decreased in the presence of increasing amounts of the competitor plasmid pBS-GAPDH-λ (upper band). To determine the exact copy numbers of GAPDH, first the radioactivity in the normal and the competitor PCR products was scanned densitometrically. Second, since the specific activities of the competitor and normal PCR products are different because of their size difference, the scanning units were normalized to the size of the competitor. Third, the normalized scanning units were plotted against the number of copies of the competitor template in the PCR. A representative graph for GAPDH quantitation in the T47D cell line is shown in Fig. 3. As seen in Fig. 3, the cross-over point at which the competitor and the reverse-transcribed cDNA gave equal
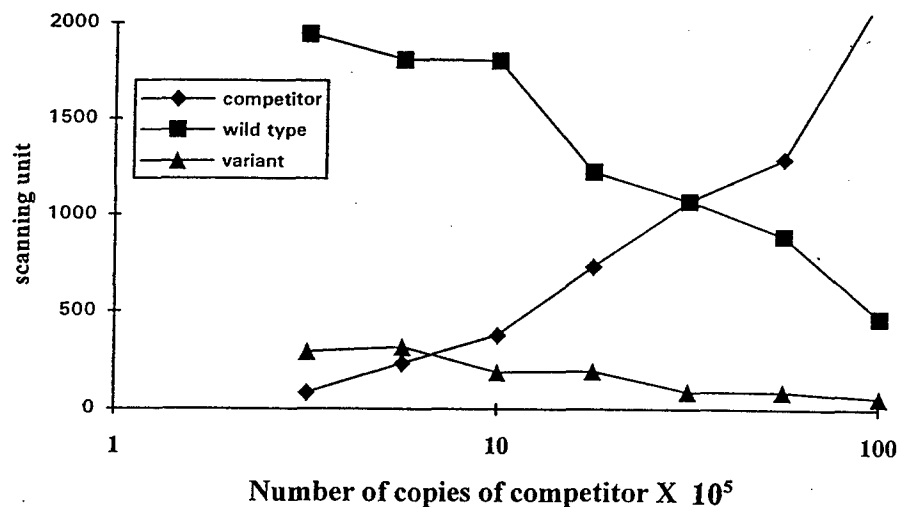
FIG. 5. Absolute quantitation of ER copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The normalized scanned units were plotted against the number of copies of the competitor pSG-ER-λ in the PCR. The cross-over points are shown at which the specific ER wild-type and exon 7Δ variant PCR products generated from T47D cDNA and competitor DNA are equal. The number of copies of ER wild-type and variant transcripts is equal to the number of copies of the competitor at the respective cross-over points.

TABLE 1

| No. | Cell type/ tumor | Copies of ER mRNA/ 10^5 copies of GAPDH | | Ratio of exon 7Δ variant to wild type |
		Wild type	Exon 7Δ variant	
1.	T47D	5.0×10^5	1.0×10^5	0.2
2.	MCF-7	4.8×10^4	1.5×10^4	0.31
3.	ZR-75	1.1×10^4	4.9×10^3	0.44
4.	Tumor 1	2.2×10^4	1.5×10^4	0.68
5.	Tumor 2	$<6.0 \times 10^3$	$<6.0 \times 10^3$	~1.00

amounts of PCR products was 1.25×10^5 copies. Therefore, the number of copies of GAPDH in T47D cDNA prepared from 50 ng of total RNA was 1.25×10^5 copies. The number of copies of GAPDH from MCF-7, ZR-75, tumor 1, and tumor 2 was determined by similar procedures. They were 7. 3×10^5, 4.3×10^5, 8.1×10^4, and 3.8×10^4, respectively, per 50 ng of reverse-transcribed total RNA. The values in the tumor samples are about 10 times lower than the tissue culture cell lines for the same amount of reverse-transcribed total RNA. The reasons for the lower values in tumor samples are not clear. This observation, however, validates the importance of normalizing the ER values to GAPDH in tumor samples instead of relying only on the RNA concentration.

A typical quantitation assay for the ER wild-type and exon 7Δ variant copy numbers using the reverse-transcribed cDNA prepared from the T47D cell line is presented in Figs. 4 and 5. The amount of competitor used in the PCR ranged from 0.56×10^5 copies (Fig. 4, lane 2) to 100×10^5 copies (Fig. 4, lane 11). As seen in Fig. 4, the ER wild-type and exon 7Δ variant products (middle and lower bands, respectively) from the T47D cell line decreased in the presence of increasing amounts of the competitor plasmid pSG-ER-λ (upper band). To determine the exact copy numbers of ER wild-type and the exon 7Δ variant, the densitometric scans of the radioactivity were normalized as in the case of GAPDH and the normal and the competitor PCR products were plotted against the number of copies of the competitor template in the PCR. A representative graph for the ER wild-type and exon 7Δ variant quantitation in the T47D cell line is shown in Fig. 5. As seen in Fig. 5, the wild-type ER and competitor templates generated equal amounts of PCR products when the competitor concentration was at 31.6×10^5 copies. The exon 7Δ variant and the competitor products crossed over when the competitor concentration was 6.6×10^5 copies. Thus, the numbers of copies of ER wild-type and exon 7Δ variant in T47D cDNA prepared from 250 ng of reverse-transcribed total RNA were 31.6×10^5 and 6.6×10^5, respectively. The numbers of copies of the wild-type and the exon 7Δ variant in MCF-7, ZR-75, and tumor tissues were determined by

similar procedures. The numbers of copies of both the wild-type and exon 7Δ variant in all the cell lines and tumor tissues studied were normalized to 10^5 copies of GAPDH. The results are presented in Table 1. The numbers were found to be repeatable in multiple experiments. Our results indicate that the T47D cell line expresses the highest amounts of ER transcripts among the cell lines studied. The results presented in Table 1 also show that the ratios of the variant to wild type are highest in ZR-75 (0.44) and lowest in T47D (0.2) among the cell lines studied. The ratios of the exon 7Δ variant to wild type in the tumor tissues are considerably higher (0.68 and ~1 in tumors 1 and 2, respectively) than in the cell lines studied. The significance of this higher variant to wild-type ratio in tumor tissues is not known. It was previously shown that the exon 7Δ variant forms a heterodimer with the wild-type protein and exerts a dominant negative effect due to constitutive DNA binding activity in the absence of the hormone. It was also suggested that the ratio of variant to wild type influences the response to antiestrogen therapies (19). However, it is not known whether the increase in the copy numbers of this variant is responsible for the genesis and progression of breast cancers.

The results presented in this report clearly establish the feasibility of determining the exact mRNA copy numbers of the ER wild type and variants in tumor tissues. Since the ER competitor we have prepared has the insert in exon 4, it can be used with any set of primer pairs which encompasses exon 4. The quantitation procedures described here will highly facilitate establishing an index between the mRNA copy numbers of ER wild- and variant-type transcripts per a given number of copies of GAPDH transcripts in the tumors and response to antiestrogen therapies.

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Quantitation of alternatively spliced estrogen receptor alpha mRNAs as separate gene populations.

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Estrogen receptor (ER) mRNA undergoes alternative splicing generating transcripts that have deletions in various combination of exons. Although several reports have shown that the spliced variant mRNAs are expressed in both normal and malignant tissues, the exact functional role(s) of these molecules have not been established in estrogen induced signal transduction processes mainly due to practical limitations involved in their detection and quantitation. We have recently described a 'Splice Targeted Primer Approach' that can specifically detect splice variants without amplifying the wild type ERs [12]. In the current report, we describe strategies to quantify individual splice variant mRNAs as separate gene populations independent of wild type or other variants using ER exon 7Delta and exon 2Delta as models. We describe the methods of quantifying the exon 7Delta and exon 2Delta transcripts in two breast cancer cell lines, MCF-7 and LCC2, and a breast tumor using the splice-targeted primers in combination with template competition RT PCR. The exon 2Delta splice specific sense primer along with an anti-sense primer in exon 4 amplified a 412 bp product in both cell lines and the tumor that could be quantitated. The exon 7Delta splice targeted anti-sense primer along with a partner primer in exon 2 amplified four transcripts that have deletions in exon 7, exons 7 and 4, exons 7 and 3-4, and exons 7 and 3-5. These four transcripts could be simultaneously quantified by the template competition method described here. Our results also show that the estrogen-independent LCC2 cells express significantly higher levels of the above 7Delta transcripts compared to the estrogen-dependent MCF-7 cells.

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Quantitation of alternatively spliced estrogen receptor alpha mRNAs as separate gene populations☆

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Abstract

Estrogen receptor (ER) mRNA undergoes alternative splicing generating transcripts that have deletions in various combination of exons. Although several reports have shown that the spliced variant mRNAs are expressed in both normal and malignant tissues, the exact functional role(s) of these molecules have not been established in estrogen induced signal transduction processes mainly due to practical limitations involved in their detection and quantitation. We have recently described a 'Splice Targeted Primer Approach' that can specifically detect splice variants without amplifying the wild type ERs [12]. In the current report, we describe strategies to quantify individual splice variant mRNAs as separate gene populations independent of wild type or other variants using ER exon 7Δ and exon 2Δ as models. We describe the methods of quantifying the exon 7Δ and exon 2Δ transcripts in two breast cancer cell lines, MCF-7 and LCC2, and a breast tumor using the splice-targeted primers in combination with template competition RT PCR. The exon 2Δ splice specific sense primer along with an anti-sense primer in exon 4 amplified a 412 bp product in both cell lines and the tumor that could be quantitated. The exon 7Δ splice targeted anti-sense primer along with a partner primer in exon 2 amplified four transcripts that have deletions in exon 7, exons 7 and 4, exons 7 and 3–4, and exons 7 and 3–5. These four transcripts could be simultaneously quantified by the template competition method described here. Our results also show that the estrogen-independent LCC2 cells express significantly higher levels of the above 7Δ transcripts compared to the estrogen-dependent MCF-7 cells. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Estrogen receptor alpha; Splice variants; Splice targeted primers; mRNA quantitation; Template competition RT PCR

1. Introduction

One of the distinguishing characteristics of estrogen receptor (ER) is that both α and β isoform mRNAs undergo alternate splicing, generating transcripts that have deletions in various combination of exons. The presence of ERα mRNAs that have deletions in exon 2-, 3-, 2–3, 4-, 5-, 6-, and 7 have been described in breast cancer cell lines, and normal and malignant tissues [1–4]. Although the exact functional role(s) of the splice variants have not been established, several lines of evidence as described below suggest that these molecules may be involved in estrogen-

induced signal transduction processes. There is a strong evidence to show that some of the splice variant mRNAs of both α and β isoforms are translated into functionally active proteins. For example, the predicted protein products of ERα exon 5Δ variant and a protein corresponding to transcript that has exon 6 and 7 duplication have been detected naturally [5,6]. Recently, Fuqua and others described the presence of ERβ splice variant proteins in breast cancer cell lines and tumors [7]. These and the results of several other studies are consistent with the ability of ER variant mRNAs to be stably translated in vivo and, therefore, may have a functional role(s). The functional properties of some of the variants have been evaluated in vitro using yeast expression system. It was found that the spliced variants have different transcriptional properties compared to wild type. For example, exon 5Δ variant activates transcription in a constitutive manner in the absence of hormone [8], and the most abundant, naturally occurring 7Δ variant forms a heterodimer with the wild type receptor and exerts a dominant negative

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effect due to constitutive DNA binding activity in the absence of the hormone [9]. A report by Klotz and others have shown that differential expression of exon 5 and 7 deletion transcripts correlate with the estrogen responsiveness in breast cancer cell lines [10]. All the above reports suggest that the levels of alternatively spliced ER molecules correlate with response to estrogens/anti-estrogens. Several other reports have shown that the expression of certain exon deletion transcripts is deregulated during breast tumorigenesis. For example, it was shown that the exon 5 deletion transcript was significantly elevated in ER⁻ and PgR⁺ breast tumors [8] and the expression of exon 3 deleted mRNA is reduced in breast tumors compared to normal tissues [11]. To conclusively establish the role(s) of these naturally occurring ER isoforms in biology and medicine, highly specific and sensitive methods are needed that can qualitatively and quantitatively investigate their expression levels in normal and neoplastic cells.

Conventionally, the ER exon deletion variant transcripts are characterized by co-amplification with the wild type sequences using reverse transcription polymerase chain reaction (PCR) (RT-PCR) approaches that by virtue of specific primer design are focused on small regions of the known wild type mRNA. However, there are several practical limitations to this approach of detection. Firstly, the threshold of detection, because the wild type transcripts are present in large excess to alternatively spliced molecules, a competitive amplification occurs among the wild type and all the alternatively spliced transcripts. Detection of products corresponding to alternatively spliced molecules depends upon the relative expression levels of their mRNA species with in the sample. Thus, spliced transcripts expressed at low levels may fall below the threshold of detection. Secondly, this approach cannot distinguish those mRNAs with multiple deletions in distant exons. For example, an ER transcript that has deletions in exons 4- and 7 cannot be distinguished from transcripts having single deletions in exon 4 or exon 7 by this method, and finally transcripts with similar sized deletions cannot be distinguished by gel exclusion chromatography.

To circumvent all the above described limitations, we have developed a novel 'Splice Targeted Primer Approach' to specifically detect the alternatively spliced molecules without amplifying the wild type receptors. This involves the targeted amplification of the alternatively spliced molecules as separate gene populations using specific primers designed at the alternative splice junctions. We have shown that the targeted primers that have a minimum of three of four bases at the extreme 3' end unique for the splice junction will specifically amplify the spliced junction without amplifying the flanking wild type exons and to design such a primer, the overhang sequences can extend up to eight bases past the splice junction [12]. In the current study, we describe strategies to quantify the absolute copy numbers of ERα splice variant mRNAs by applying Splice Targeted Primers in combination with RT PCR Template Competition approach using exon 7Δ and exon 2Δ mR-

NAs as models. The significance of our approach is that the individual ER splice variant levels could be accurately measured as separate gene populations independent of wild type.

2. Experimental

2.1. RNA Isolation and cDNA preparation

The total RNA from breast cancer cell lines and a breast tumor was isolated using Trizol Reagent as described before [13]. The cDNA was prepared from the total RNA using Omniscript cDNA synthesis kits from QIAGEN. Briefly, the standard mixture contained one μg of total RNA, 10 U of RNase inhibitor, 0.5 mM each of dNTPS, 1-μM random hexamers and 4 U of Omniscript reverse transcriptase in a total volume of 20 μl. For reverse transcription, first the tubes were incubated at 37°C for 60 min followed by 95°C for 5 min and finally rapid cooling on ice.

2.2. Construction of competitor plasmids for the quantitation of exon 2Δ and 7Δ transcripts by Template Competition

In the template competition method of mRNA quantitation, the competitor plasmid is constructed with an extra fragment of an irrelevant DNA sequence internal to the PCR primer location that generates a larger PCR product than the template of interest and distinguishable by size exclusion gel electrophoresis [14]. The construction of the competitors was accomplished as follows: For the construction of exon 7Δ competitor, first, its cDNA was amplified using splice targeted anti-sense primer, ER AX6/8, 5' CTCCATGCCTTTGTTA 8/6 CAGAA 3' (positions, exon 8, bp 1801 to 1786 and exon 6, bp 1601–1597), and a sense primer, ER1S, 5' TGCCCTACTACCTG-GAGAACG 3' (position, exon 1, bp 615–635) by RT PCR from a breast cancer cell line, LCC1. The sequence and locations of all the primers described here are based on ERα cDNA sequence published by Green and others [15]. The above primers specifically amplified an expected 1 kb length fragment, which was confirmed by sequence analysis as ER exon 7Δ, without amplifying the wild type cDNA. Second, the 1kb exon 7Δ product was gel purified and cloned into pCR®II-TOPO (Invitrogen, San Diego, CA, USA) vector according to manufacturer's directions. Third, the pCR®II-TOPO-ER7Δ generated as above was used to construct the competitor plasmid in two steps: 1) pCR®II-TOPO-ER7Δ was digested with _EcoRI_ to release the cloned ER 7Δ fragment from two _EcoRI_ sites that flank the insert in pCR®II-TOPO and subcloned into an unique _EcoRI_ site in the vector, pSG5 (Strategene, La Jolla, CA, USA) and 2) a 366 bp fragment of DNA from bacteriophage λ was amplified as described before [16] and inserted into an unique _HindIII_ site in exon 4 of ER 7Δ fragment of the plasmid, pSG5-ER7Δ. The resulting competitor was termed as pSG5-ER7Δ- λ. Similarly, to construct a competitor for exon 2Δ quantitation, a 412 bp exon 2Δ cDNA was amplified using
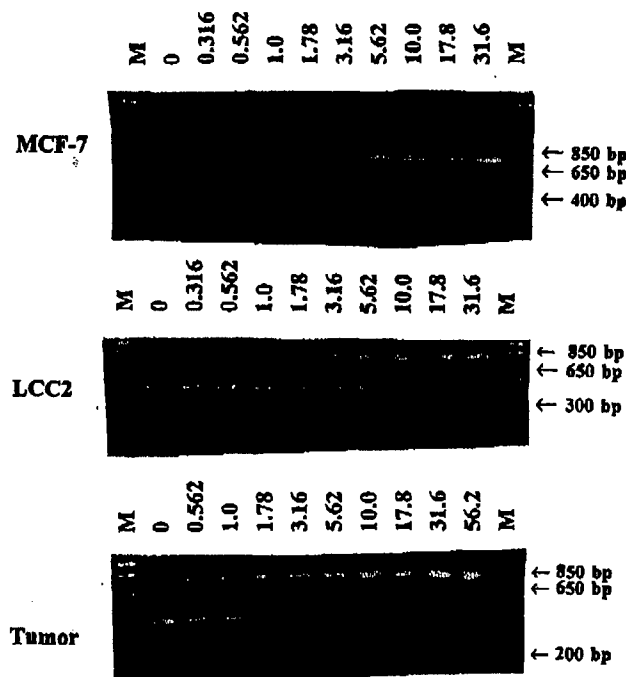
NUMBER OF COPIES OF COMPETITOR X 10⁴

Fig. 1. Quantitation of ER exon 2Δ copy numbers in two breast cancer cell lines, MCF-7, LCC2 and a breast tumor. Template competition assay was performed with constant amount of cDNA, prepared by reverse transcription of 250 ng of total RNA, plus varying amounts of the competitor plasmid, pSG5-ER2Δ- λ and the primers ER SX1/3 and ERA as described in the text. The amplified products (5 µl) were electrophoresed in 1.2% agarose gels and detected by ethidium bromide staining. The decrease in the 412 bp ER 2Δ product from the cell lines and the tumor (lower band) is seen in the presence of increasing amounts of the competitor, pSG5-ER2Δ- λ (778 bp) product (upper band).

splice targeted sense primer, *ER SX1/3*, 5′ CGCCGGCATTC-TACAG 1/3 GACAT 3′ (positions, exon 1, bp 669–684 and exon 3, bp 876–880), and an anti-sense primer, ERA, 5′ GGTCAGTAAGCCCATCATCG 3′ (position, exon 4, bp 1272–1254) by RT PCR from LCC1 and cloned into pCR®II-TOPO vector. The 412 bp exon 2Δ was subcloned into pSG5 and a 366 bp λ fragment was inserted into the HindIII site in exon 4 as described above to generate pSG5-ER2Δ-λ.

2.3. Competition assays

The pSG5-ER2Δ- λ and the pSG5-ER7Δ- λ were used in the competition assays to determine the absolute copy numbers of ER exon 2Δ and ER exon 7Δ mRNAs in two breast cancer cell lines, MCF-7 and LCC2, and a breast tumor by template competition RT PCR approach. The template competition assay was performed with cDNA prepared by reverse transcription of the total RNA and competitor plasmids. The PCRs were carried out with a constant amount of cDNA (reverse tran-

scribed from 250 ng of total RNA) plus varying amounts of competitor plasmids (0–310.0 × 10⁵ copies in quarter-log increments). The number of copies of the competitor DNA molecules were determined by multiplying the average mass of a base pair (660 Da) by the number of base pairs of the competitor and then dividing Avagadro's number by that value. The PCRs were performed in 25 µl volume using 2 µm each of the sense and anti-sense primers, 1× Q solution (Qiagen, Chatsworth, CA, USA), 1× PCR buffer, 200 µm each of dNTPs, and 0.6 U of Taq polymerase. For the amplification of exon 2Δ, the primers ER SX1/3 and ERA were used and for exon 7Δ transcripts, ER AX6/8 and ER2S (5′ AAGGCCT-TCTTCAAGAGAAG 3′) (position, exon 2, bp 848–867) were used. The PCR conditions were initial denaturation for 5 min at 95°C followed by 94°C for 1 min, annealing for 1 min at 55°C, and extension for 2 min at 72°C for 40 cycles and final extension for 10 min at 72°C. The PCR products (5.0 µl) were electrophoresed in 1.2% agarose gels and visualized by ethidium bromide staining. To determine the exact copy num-

3. Results and discussion

Several studies have shown that estrogen-responsive tissues such as breast, ovary, uterus, bone, and brain express the splice variants of ERα and ERβ in addition to the wild type receptors. In vitro studies with individual receptors have demonstrated that the α and β and the alternatively spliced variants of ERα have distinct ligand binding and transcriptional properties [8,9, 17,18]. It strongly follows that the relative proportion of various receptors expressed in a tissue will result in different ligand- and DNA binding properties. In all likelihood, the response to a particular estrogen/anti-estrogen depends on the relative amounts of all the ER isoforms. Therefore, the ability to precisely quantify all the ER isoforms will significantly contribute to our understanding of the complex biology governed by natural estrogens and in designing tissue specific synthetic estrogens and anti-estrogens. Our goal has been to define the properties of the total represented by each of the ER isoforms. As a first step in pursuit of that goal, we have begun develop- ing molecular assays that can precisely quantify every isoform of the ER. We have recently described molecular methods for the quantification of exact copy numbers of wild type ERα mRNA in both tissue culture cells [16] and a novel 'splice targeted primer approach', that can detect every possible alternatively spliced isoform of ERα [12]. As shown in the

results below, the approach we have developed can precisely quantitate the alternatively spliced mRNAs independent of wild type in breast cancer cell lines as well as breast biopsy samples. Furthermore, the described method can quantify sin- gle and several multiple exon deletion transcripts simulta- neously.

A typical quantitation assay performed for ER 2A and exon 7A in MCF-7 and LCC2 cells and a breast tumor by template competition method is shown in Figs. 1 and 3. In the template competition method, the two templates, the cDNA of interest and the competitor, are amplified by the same primers used in the PCR. Therefore, the ratio of their PCR products will reflect the starting ratio of the two template DNAs. This means that if the amount of competitor is known, then the amount of the transcript of interest can be precisely determined by measuring the two PCR products. As seen in Fig. 1, the amount of 412 bp ER 2A product from the cell lines and the tumor (lower band) decreased in the presence of increasing amounts of the com- petitor, pSG5-ERΔ2-A (778 bp) product (upper band).

To determine the exact copy numbers of exon 2A, first, the fluorescence intensity in the normal and the competitor PCR products was scanned densitometrically. Second, be- cause the competitor and the normal PCR products are of different sizes, the fluorescence intensity of exon 2A prod- uct was normalized to the size of the competitor. This was accomplished by multiplying the scanning units of exon 2A with a factor (1.88) that was obtained by dividing the num- ber of bases of the competitor (778) by the number of bases of exon 2A (412). Next, the normalized scanning units of exon 2A and the competitor scanning units were plotted against the number of copies of the competitor template in the PCR reaction. A representative graph for the quantita-
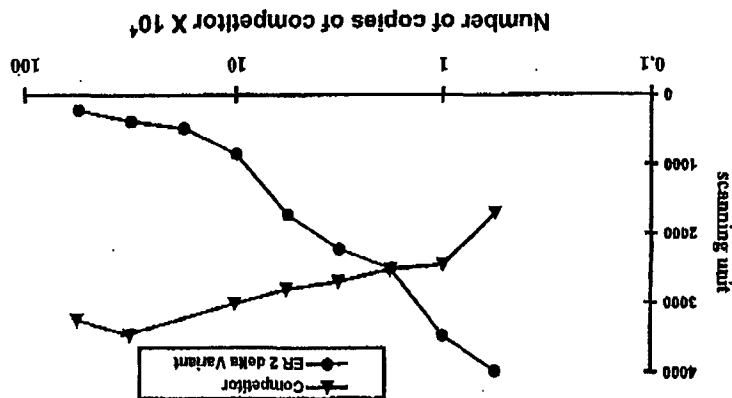
Fig. 2. Absolute quantitation of ER exon 2A transcripts in a breast tumor tissue. The intensity of the PCR products in the tumor panel in Fig. 1 was measured by scanning the photographs of the ethidium bromide stained gels in a Mustang 1200–3E scanner and quantitated using NIH Image 1.60 program. The scanning units of exon 2A were normalized to the size of the competitor and the normalized scanning units were plotted against the number of copies of the competitor, pSG5-ERΔ2-A, in the PCR reaction. The cross-over point is shown at which the specific ER 2A product generated from the breast tumor and the competitor DNA are equal. The number of copies of ER 2A in 250 ng of total RNA is equal to the number of copies of the competitor at the cross-over point (1.5×10^4).

Number of copies of competitor X 10^4

Legend: Competitor; ER 2 delta Variant

(y-axis: scanning unit — 0, 1000, 2000, 3000, 4000; x-axis: 0.1, 1, 10, 100)
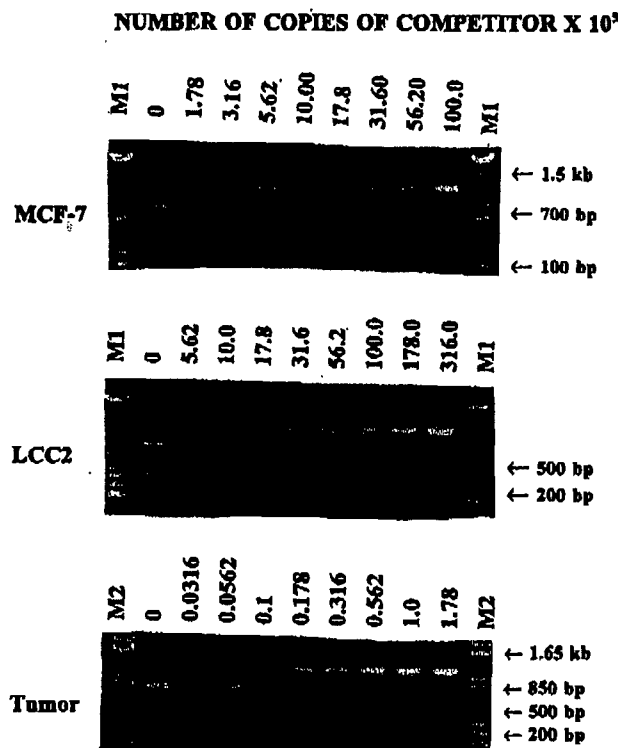
NUMBER OF COPIES OF COMPETITOR X 10⁵

Fig. 3. Quantification of ER exon 7Δ mRNA copy numbers in two breast cancer cell lines, MCF-7 and LCC2 and a breast tumor. Template competition assay was performed with constant amount of cDNA, prepared by reverse transcription of 250 ng of total RNA, varying amounts of the competitor plasmid, pSG5-ER7Δ- λ, and the primers ER AX6/8 and ER2S as described in the text. The amplified products were electrophoresed in 1.2% agarose gels and detected by ethidium bromide staining. The four bands with sizes, 769 bp, 433 bp, 316 bp, and 177 bp in the absence of any competitor (0 copies) in both cell lines and the tumor were identified as transcripts that have deletions in exon 7, exons 7 and 4, exons 7 and 3–4 and exons 7 and 3–5, respectively by sequence analyses.

tion of exon 2Δ in the breast tumor is shown in Fig. 2. As seen in the figure, the cross-over point at which both the competitor and the exon 2Δ from the tumor cDNA, reverse transcribed from 250 ng of total RNA, gave equal amounts of PCR products was 1.84×10^4 copies. Therefore, the number of copies of ER 2Δ in the breast tumor cDNA, prepared from 250 ng of total RNA, was 1.84×10^4 copies. Similarly, the cross-over points in MCF-7 and LCC2 cell lines were 7.6×10^4 and 15×10^4, respectively. These numbers were repeatable in four experimental trials.

Quantitation of exon 7Δ transcripts in MCF-7, LCC2 and a breast tumor is shown in Fig. 3. The primers ERAX6/8 and ER2S amplified four products of sizes 769 bp, 433 bp, 316 bp, and 177 bp in both cell lines and the tumor. They were identified as transcripts that have deletions in exon 7, exons 7 and 4, exons 7 and 3–4 and exons 7 and 3–5, respectively by sequence analyses. Fig. 3 shows that the amounts of the above single-, double-, triple and quadruple exon deletion variants decreased in the presence of increasing amounts of pSG5-

ER7Δ- λ, To determine the exact copy numbers of all exon 7Δ mRNAs, the scanning units of all four bands were normalized to the size of the competitor as described above and the normalized scanning units and the competitor scanning units were plotted against the number of copies of the competitor template in the PCR reaction. A representative graphs for the quantitation of all four variants in the breast tumor are shown in Fig. 4, panels A-D. As seen in the figure, the cross-over points for the single-, double-, triple-, and quadruple exon deletion variants were 1×10^4 copies, 7.5×10^3 copies, 1.7×10^4 copies and 4.8×10^4 copies, respectively. Although the amounts of the multiple exon deletion variants seem less than the single exon deletion variant in Fig. 3, after normalizing the scanning units for the sizes, their levels are equal or near equal to the single deletion variant. The amounts of exon 2Δ and exon 7Δ variants in both cell lines and the tumor are summarized in Table 1.

The results described here also show that alternatively spliced variants that have deletions in various combination of exons are present in both tissue culture cell lines
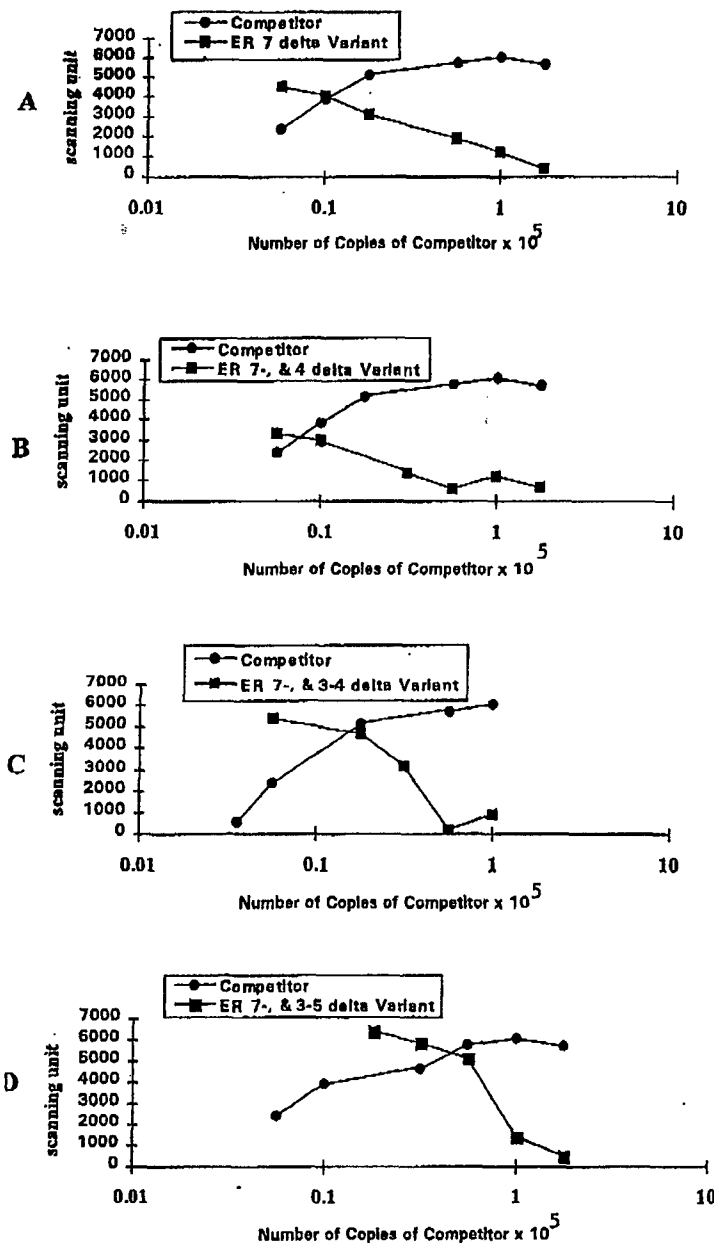
Fig. 4. Absolute quantitation of various ER 7Δ copy numbers in a breast tumor tissue. The intensity of the PCR products in the tumor panel in Fig. 3 was quantified by first scanning the photographs of the ethidium bromide stained gels in a Mustaq 1200–3B scanner and quantitated using NIH Image 1.60 program. The scanning units of exon 7Δ, exons 7Δ and 4Δ, exons 7Δ and 3–4Δ, and exons 7Δ and 3–5Δ products in every lane were first normalized to the size of the competitor. The normalized scanning units were plotted against the number of copies of the competitor, pSG5-ER7Δ- λ, in the PCR reaction. The panels A, B, C and D show the plots for exon 7Δ, exons 7Δ and 4Δ, exons 7Δ and 3–4Δ, and exons 7Δ and 3–5Δ products, respectively. The cross-over points for the above four products were 1×10^4, 7.5×10^3, 1.7×10^4, and 4.8×10^4, respectively. The number of copies of the four 7Δ variants in 250 ng of total RNA are equal to the number of copies of the competitor at their respective cross-over points.

Table 1
Copy numbers of ER exon 2Δ and 7Δ transcripts per microgram of total RNA

No.	Cell Type;Tumor	2Δ	7	7Δ & 4Δ	7Δ & 3–4Δ	7Δ & 3–5Δ
1.	McF-7	3.06×10^5	1.8×10^6	1.24×10^6	1.44×10^6	1.8×10^6
2	LCC2	6.0×10^5	4.0×10^6	2.44×10^6	1.08×10^7	4.08×10^7
3.	Tumor	7.2×10^4	4.0×10^4	3.0×10^4	6.8×10^4	1.92×10^5

and tumors. The data in Table 1 show a 2 to 10-fold difference in the expression levels of exon 7Δ transcripts between the estrogen-dependent MCF-7 cells and the estrogen-independent cell line, LCC2, which is also resistant to the anti-estrogen, Tamoxifen [19]. As stated earlier, exon 7Δ variant lacks the ligand binding property and exerts a negative dominant effect. The double, triple and quadruple exon deletion variant transcripts lack both ligand binding and the entire hinge regions. These transcripts could result in transcriptionally active proteins, over expression of which could contribute to estrogen independence. It is possible that the LCC2 cells are estrogen-independent due to the increased levels of the above four transcripts. We expect that the strategies described here for the quantitation of various alternatively spliced ER mRNAs will be highly helpful in generating data that will throw light on hormone independence in conditions such as breast cancer. Quantitative analysis of various ER splice variants in different estrogen responsive normal- and malignant tissues may also prove these molecules to be more important than currently considered in understanding the tissue specific actions of natural estrogens and in designing synthetic estrogens and anti-estrogens.

The above described template competition RT PCR in combination with splice targeted primers could be applied for the amplification and quantitation of exact mRNA copy numbers of all ERα splice variants except ER 4Δ mRNA, because it lacks the unique *HindIII* site to construct a competitor in the above manner. A competitor for ER 4Δ, however, can be constructed by inserting λ sequence into an unique *BglII* site in exon 6. The results described here also show that depending upon the position of the partner primer chosen, the splice targeted primers can amplify several related transcripts that have deletions in various combination of exons that could be simultaneously quantitated.

Acknowledgments

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Primer Design Strategies for the Targeted Amplification of Alternatively Spliced Molecules

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The alternatively spliced mRNAs are conventionally detected by RT–PCR approaches, which by virtue of specific primer design, are focused on small regions of the known wild-type mRNA. However, there are major problems in this approach, such as: (1) threshold of detection—a competitive amplification occurs among the wild-type and all the alternatively spliced molecules; therefore, the detection of a particular spliced molecule depends on the relative expression levels of its mRNA species within a sample; and (2) the mRNAs which have multiple deletions in distant exons such as an estrogen receptor (ER)[2] transcript which has deletions in exons 2 and 7 cannot be distinguished from transcripts having single deletions in exon 2 or 7 by this method (1).

To circumvent these problems, Yang and Le (2) described specific amplification of alternatively spliced molecules using targeted primers which have three of three unique overhang bases across the splice junction. However, in many instances there is often a complete or partial homology between the bases in the wild-type junctions and the alternate splice junctions. In these cases, it is necessary to target sequences beyond the first three bases of the splice junction to identify the unique bases. In the current study, we wanted to determine the number of extreme 3′ bases in the primer that were required to be unique and the number of bases the primer could extend beyond the junction and still maintain specific amplification of the target sequences. We designed and tested a series of primers using ER exons 7Δ, 5Δ, 2Δ, and 2–3Δ as models with plasmids containing their respective nucleotide sequences and full-length wild-type ER cDNA, pIC-ER-F. The construction of ER plasmids containing the exon

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[2] Abbreviations used: ER, estrogen receptor; exon Δ, exon deletion; AX, antisense; SX, sense.

7-, 5-, 2-, and 2–3Δ sequences was accomplished as follows. First, their respective nucleotide sequences were coamplified with wild-type sequences using the cDNA prepared from the cell line MCF-7 (3) and cloned into pCRII-TOPO vector. The exon 7Δ sequence (688 bp) was amplified using a sense primer, ER1S, 5′-GCCCGCTCATGATCAAACGC-3′ (position: exon 4, bp 1113–1132), and an antisense primer, ER1A, 5′-TACTTTTGCAAGGAATGCGA-3′ (position: exon 8, bp 1977–1958), and cloned to obtain pCRII-TOPO-7Δ. The sequence and locations of all the primers described here are based on the ER cDNA sequence published by Green and others (4). The exon 5Δ nucleotide sequence (300 bp) was amplified using a sense primer, ER2S, 5′-GGAGACATGAGAGCTGCCAAC-3′ (position: exon 4, bp 1082–1102), and an antisense primer, ER2A, 5′-CCAGCAGCATGTCGAAGATC-3′ (position: exon 6, bp 1520–1501), and cloned to obtain pCRII-TOPO-5Δ. The exon 2Δ (468 bp) and exons 2–3Δ (350 bp) sequences were generated using the sense primer, ER3S, 5′-TGCCCTACTACCTGGAGAACG-3′ (position, exon 1, bp 615–635), and an antisense primer, ER3A, 5′-GGTCAGTAAGCCCATCATCG-3′ (position: exon 4, bp 1273–1254). The resulting exon 2Δ and exon 2–3Δ plasmids were termed as pCRII-TOPO-2Δ and pCRII-TOPO-2–3Δ, respectively. The PCRs were carried out using 50 ng of the test plasmid at initial denaturation conditions of 5 min at 95°C followed by 94°C for 1 min, annealing for 1 min at either 61 or 68°C as indicated in the text, and extension for 2 min at 72°C for 30 cycles plus a final extension for 10 min at 72°C.

A series of antisense primers (as illustrated in Table 1), ER AX6/8-1 (positions: exon 8, bp 1804–1786, and exon 6, bp 1601–1600) through ER AX6/8-7 (positions: exon 8, bp 1798–1786, and exon 6, bp 1601–1594) which have varying number of overhanging bases at the 6/8 splice junction (Table 1A) were tested together with the sense primer, ER1S, at an annealing temperature of 61°C. If the targeted primers specifically recognize only the 6/8 splice junction, we would expect them to generate a PCR product ranging from 508 bp for ER AX6/8-1 to 502 bp for ER AX6/8-7 with pCRII-TOPO-7Δ and no product with pIC-ER-F. On the other hand, if the exon 8 portion of the primers anneals and amplifies the wild-type sequences in pIC-ER-F, we would expect to observe a PCR product from 692 bp with ER AX6/8-1 to 686 bp with ER AX6/8-7. Similarly, if the exon 6 portions of the primers recognize and amplify exon 6 of the wild-type sequences in pIC-ER-F, we would expect to observe PCR products as described for the pCRII-TOPO-7Δ. The results are shown in Fig. 1A. Lanes 1–7 contain the PCR products amplified with the targeted primers ER AX6/8-1 through ER AX6/8-7, respectively, using pCRII-TOPO-7Δ as the template and lanes 8–14 contain corresponding products with pIC-ER-F as the template. As seen in lanes

TABLE 1

Design of Antisense Primers for Targeting ER Splice Variants, ER 7Δ and ER 5Δ

	Exon 8	Exon 6	Number of 3' overhang bases		Target spec annel. te:
			Total	Unique in the extreme 3' end	61°C

A. Primers for exon 7Δ

	Exon 8	Exon 6	Total	Unique	61°C
ER AX6/8-1	5' ATGCTCCATGCCTTTGTTA//	<u>C</u>**A** 3'	2	1/1	No[a]
ER AX6/8-2	5' TGCTCCATGCCTTTGTTA//	<u>C</u>**AG** 3'	3	2/2	No[a]
ER AX6/8-3	5' GCTCCATGCCTTTGTTA//	<u>C</u>**AGA** 3'	4	2/3	No[a]
ER AX6/8-4	5' CTCCATGCCTTTGTTA//	<u>C</u>**AGAA** 3'	5	3/4	Yes
ER AX6/8-5	5' TCCATGCCTTTGTTA//	<u>C</u>**AGAAT** 3'	6	4/5	Yes
ER AX6/8-6	5' CCATGCCTTTGTTA//	<u>C</u>**AGAATT** 3'	7	4/6	Yes
ER AX6/8-7	5' CATGCCTTTGTTA//	<u>C</u>**AGAATTA** 3'	8	5/7	Yes
Wild type	5 TGCCTTTGTTA//	<u>C</u>TCATGTG 3'			
	Exon 8	Exon 7			

B. Primers for Exon 5Δ

|	Exon 6	Exon 4	Total	Unique	61°C
ER AX4/6-1	5' CACATTTTCCCTGGTTC//	<u>C</u>TG**G** 3'	4	1/1	No[a]
ER AX4/6-2	5' ACATTTTCCCTGGTTC//	<u>C</u>TG**GC** 3'	5	1/2	No[a]
ER AX4/6-3	5' CATTTTCCCTGGTTC//	<u>C</u>TG**GCA** 3'	6	2/3	No[a,b]
ER AX4/6-4	5' ATTTTCCCTGGTTC//	<u>C</u>TG**GCAC** 3'	7	3/4	No[b]
ER AX4/6-5	5' TTTTCCCTGGTTC//	<u>C</u>TG**GCACC** 3'	8	4/5	No[b]
ER AX4/6-6	5' TTTCCCTGGTTC//	<u>C</u>TG**GCACCC** 3'	9	5/6	No[b]
ER AX4/6-7	5' TTCCCTGGTTC//	<u>C</u>TG**GCACCCT** 3'	10	6/7	No[b]
Wild type	5' CCCTGGTTC//	<u>C</u>TG**TCCAAGA**..3'			
	Exon 6	Exon 5			

Note. N/T, not tested.

[a] Amplifies the wild-type exon at the 5' end of the primer.

[b] Amplifies the wild-type exon at the 3' end of the primer.

[c] Not applicable.

1–7, all the targeted primers generated the expected ~500-bp product with pCRII-TOPO-7Δ. However, when the template was pIC-ER-F, the targeted primers ER AX6/8-1 through ER AX6/8-3, which have fewer than three bases unique to the splice junction in the extreme 3' end, generated an approximately 690 bp PCR product (lanes 8–10), indicating the annealing of these primers to exon 8 of the wild-type ER sequences. On the other hand, the primers ER AX6/8-4 through ER AX6/8-7, all of which have a minimum of three or more bases unique to the splice junction in the extreme 3' end, did not generate any PCR products with pIC-ER-F (lanes 11–14). These results indicated the absence of amplification by their annealing to either exon 7/8 or 6/7 junctions in the wild type. Based on these results, it can be hypothesized that the ER 7Δ targeted primers which have a minimum of three of four unique bases at the extreme 3' end will specifically amplify the spliced junction without amplifying the flanking exons. In addition, it appears that as many as eight bases past the exon 6/8 splice junction are permitted without amplification of the wild-type sequences.

Next, we tested the above hypothesis using antisense primers, ER AX4/6-1 (positions: exon 6, bp 1484–1468, and exon 4, bp 1328–1325) through ER AX4/ (positions: exon 6, bp 1478–1468, and exon 4, bp 13 1319) (Table 1B) for the specific amplification of exon 5Δ. As seen in Table 1B, both exon 4/6 and junctions have the same initial three bases, C1 Therefore, we designed primers further beyond 1 splice junction to target the unique sequences a tested together with the sense primer, ER1S, at annealing temperature of 61°C. If the targeted prim are specific for the 4/6 junction, we would expect observe PCR products ranging from 233 bp with 1 AX4/6-1 to 227 bp with ER AX4/6-7 when amplifyi pCRII-TOPO-5Δ and no product with pIC-ER-F. Alt natively, if the targeted primers anneal and ampl exon 6 or 4 of the wild-type junctions in pIC-ER-F, v would expect to observe PCR products of sizes appro imately 370 or 230 bp, respectively. The PCR resul with these primers are shown in Fig. 1B, a. Lanes 1- contain the PCR products amplified with the target primers ER AX4/6-1 through ER AX4/6-7, respectivel with pCRII-TOPO-5Δ and lanes 8–14 contain corr sponding products with pIC-ER-F. As seen in lan 1–7, all of the targeted primers generated the expect ~230-bp product with pCRII-TOPO-5Δ. Howeve:
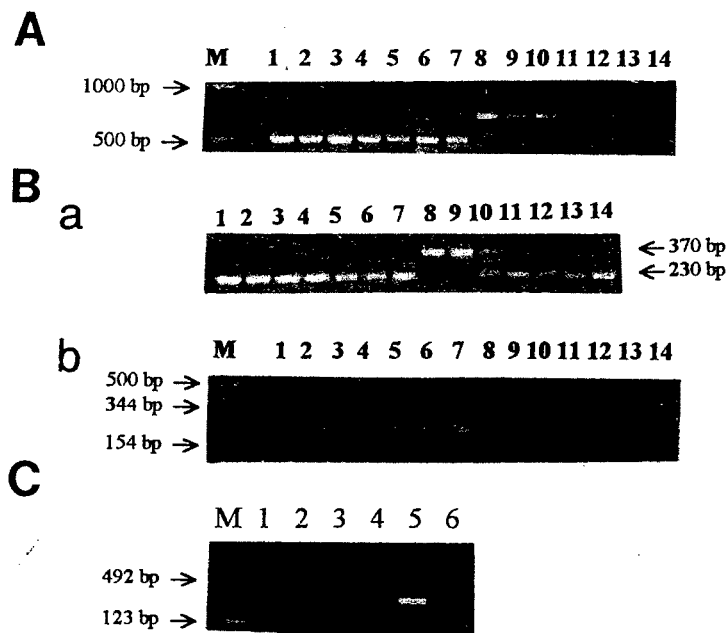
FIG. 1. Testing of targeted primers for the ER exon 6/8, 4/6, 1/3, and 1/4 splice junctions with plasmid templates containing their respective sequences and wild-type sequences. (A) Lanes 1–7 contain the PCR products amplified with the primers ER AX6/8-1 through ER AX6/8-7, respectively, using pCRII-TOPO-7Δ as the template and lanes 8–14 contain corresponding products using pIC-ER-F as the template. (B, a and b) a and b show the PCR products obtained at the annealing temperatures of 61 and 68°C, respectively, with the primers ER AX4/6-1 through ER AX4/6-7. In each of these panels, lanes 1–7 contain the PCR products amplified using pCRII-TOPO-5Δ as the template and lanes 8–14 contain the corresponding products using pIC-ER-F as the template. (C) Lanes 1–3 contain the PCR products amplified with ER SX1/3 and ER3A and using no DNA, pCRII-TOPO-2Δ and pIC-ER-F, respectively, as the templates. Lanes 4–6 contain the PCR products amplified with the primer, ER SX1/4, and ER 3A using no DNA, pCRII-TOPO-2-3Δ, and pIC-ER-F, respectively, as templates. In each of these panels, the lane M contains molecular weight standards.

when the template was pIC-ER-F, primers AX4/6-1 through AX4/6-3 generated an ≈370-bp PCR product, indicating the annealing of 5′ end of these primers to exon 6 and amplification of the wild-type ER sequences (lanes 8–10). The targeted primers, AX4/6-4 through AX4/6-7, generated an ~230-bp band with the wild-type plasmid, indicating that the 3′ end portion of these primers recognized and amplified exon 4 of the wild-type sequences (lanes 11–14). The primer ER AX4/6-3 generated both 370- and 231-bp products indicating that it amplified from both exons 4- and 6 of the wild-type sequences (lane 10). Since none of the targeted primers were completely specific at the annealing temperature of 61°C, we subsequently tested them at an annealing temperature of 68°C. The results are shown in Fig. 1B, b. The primers, ER AX4/6-2 through ER AX4/6-7 maintained their ability to amplify the targeted sequences at 68°C (lanes 2–7) with pCRII-TOPO-5Δ. The PCR results with pIC-ER-F are shown in lanes 8–14. The primer ER AX4/6-1 did not recognize either pCRII-TOPO-5Δ or the pIC-ER-F (lanes 1 and 8, respectively). The primers, ER AX4/6-2 through ER AX4/6-5 did not generate any PCR products with pIC-ER-F (lanes 9–12), indicating their specificity to 4/6 junction at an annealing temperature of 68°C. The primers AX4/6-6 and AX4/6-7 amplified a

minor product of size ~230 bp (lanes 13 and 14), indicating amplification of sequences in exon 4 of the wild type. Thus it appears that in addition to the number of unique overhang bases in the extreme 3′ end of the primer, the annealing temperatures also influence the specific amplification of the targeted sequences. The above-described results with targeted primers for ER exon 5 splice variant provide evidence for our earlier hypothesis that a minimum of three of four bases unique to the splice junction in the extreme 3′ end of the primer are required to specifically amplify the alternate splice junction. It also appears that in order to design such a primer, the overhang sequences can extend up to eight bases past the splice junction without annealing and amplification of the wild-type sequences.

Keeping these parameters in mind, we next designed the targeted primers, ER SX1/3, 5′-CGCCGGCATTC-TACAG 1/3 GACAT-3′ (positions, exon 1, bp 669–684, and exon 3, bp 876–880) and ER SX1/4 5′-GCCG-GCATTCTACAG 1/4 GGATAC-3′ (positions, exon 1, bp 670–684 and exon 4, bp 993–998), for the exons 1/3 and 1/4 splice junctions, respectively. Each of these primers satisfy the requirements of our target primer design based on the results described above. We next individually tested these two sense primers using the

antisense primer, ER3A, at an annealing temperature of 61°C. The results are shown in Fig. 1C. Lanes 1–3 contain the PCR products generated with the targeted primer ER SX1/3 and the antisense primer ER3A using no DNA, pCRII-TOPO-2Δ, and pIC-ER-F as templates, respectively. Similarly, lanes 4–6 contain the PCR products with the targeted primer, ER SX1/4, and the ER3A using no DNA, pCRII-TOPO-2-3Δ and pIC-ER-F respectively as templates. As seen in Fig. 1C, the targeted primers designed for the exon 2Δ and 2–3Δ molecules amplified only the targeted splice junctions and generated the expected 414 bp and 296 bp products (lanes 2 and 5, respectively) but did not generate any products with the ER wild-type sequences (lanes 3 and 6, respectively). Thus the targeted primers which meet the requirement of at least three of four unique bases in the extreme 3' end exclusively amplify the alternate sequences. We believe that the principles developed in the current study with ER will have broad applicability to splice variants of a diverse range of genes.

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Alterations in the estrogen receptor alpha mRNA in the breast tumors of African American women

Abstract Several recent reports have shown that the mortality rate with breast cancer is about three times higher in African American women than in other populations. In addition, the available data also indicate that the tumors are very aggressive and poorly differentiated with a very low frequency of hormone receptors. To gain an insight into the factors that may be responsible for their aggressive tumors, we investigated the transcript profiles of the estrogen receptor (ER), the most important prognostic factor in breast cancer, in the tumors derived from African American women. We analyzed 24 immunohistochemically ER^+ and 6 ER^- malignant tumors for ER mRNA by reverse transcription polymerase chain reaction using a number of primer pairs. For comparative purposes, 20 ER^+ malignant tumor issues derived from Caucasian patients were also included. Our results showed that only 15 of the ER^+ tumors from African American women patients had full-length wild-type receptor transcripts and the others exhibited alterations/truncations in exon 8. We also found that the majority of tumors that had alterations/truncations in exon 8 did not express the naturally occurring, more abundant exon 7 deletion transcript. Most of the tumors expressed exon 2, exons 2–3, and exon 5 deletion variant transcripts. Unexpectedly, 2 of the 6 immuno-histochemically ER^- tumors showed full-length wild-type receptor mRNA but none of the variant transcripts.

Key words Estrogen receptor · Exon truncations · Breast tumors · African American women

Abbreviations *ER* estrogen receptor · *RT-PCR* reverse transcription polymerase chain reaction · *Exon Δ* exon deletion

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Introduction

Recent reports indicate that the experience with breast cancer varies in different populations. The incidence of breast cancer is reported to be slightly lower in African American women than in other women (Miller et al. 1993). However, the incidence seems to differ in different age groups. It is higher in young but lower in middle-aged and older African American women than in women of similar age groups in other populations (Boring et al. 1994). Trends in survival rate also seem to differ both by age and race. It was reported that the death rate from breast cancer for African American women under the age 65 has increased, while for women in a similar age group in other populations it has declined. Death rates have increased for all women over the age 65 and the increase is reported to be three times higher in African American women than in others. Several reports indicate that this high mortality rate is not due to differences in socioeconomic status, stage of diagnosis, known risk factors or methods of treatment (Moormeier 1996). It has been hypothesized that breast tumors across racial and ethnic groups could have different biological characteristics that may account for survival disparities (Elledge et al. 1994). However, such factors have not been identified thus far. There also appear to be differences in tumor biology in different racial groups. Histochemical studies of the tumors derived from African American women have shown a higher incidence of poorly differentiated tumors and an increased frequency

of nuclear atypia, higher mitotic activity, and tumor necrosis (Chen et al. 1994). Immunohistochemical studies have also shown that the frequency of estrogen (ER) and progesterone receptor (PR) expression is significantly lower in the tumors of African American women (Gapstur et al. 1996). The presence of these receptors is generally associated with well or moderately differentiated tumors and considered a good prognosis for longer survival and lower risk of tumor recurrence. The biological factors responsible for the poorly differentiated and aggressive tumors with lower frequency of hormone receptors, which may contribute to lower survival in African American women, are not known. In the current study, we examined the tumors derived from African American women for alterations in ER structure which may, in part, contribute to the aggressive nature of these tumors. We investigated the ER mRNA by reverse transcription and the polymerase chain reaction (RT-PCR), using a number of primer pairs. Our results, described here, show that only 15 of the 25 tumors that were diagnosed as ER^+ by immunohistochemistry had detectable full-length wild-type ER mRNA and others showed alterations/truncations in exon 8. Another observation is that most of the tumors that had exon 8 alterations/truncations did not express the naturally occurring, more abundant exon 7Δ variant mRNA.

Materials and methods

AmpliTaq PCR core kits and QIAquick gel-extraction kits were from Qiagen, Inc., Santa Clara, Calif. Reverse transcriptase kits were from Applied Biosystems, Foster City, Calif. $[\alpha\text{-}^{32}P]dCTP$ (Specific activity 3000 Ci/mmol, catalog no. AA0005) was from Amersham, Piscataway, N.J. The primers for amplifying ER and glyceraldehyde-3-phosphate dehydrogenase were synthesized at Gibco-BRL Life Technologies, Rockville, Md. Trizol reagent for total RNA isolation was purchased from Gibco-BRL Life Technologies. Diethyl-pyrocarbonate-treated water was from Research Genetics, Huntsville, Ala. PCR quality water and TRIS/EDTA buffer were from Biofluids, Rockville, Md. pCRII-TOPO, a cloning vector for PCR amplified products was obtained from Invitrogen, Carlsbad, Calif.

Breast tumor samples

The breast tumor samples from African American women patients were collected from Howard University Hospital and Providence Hospital in Washington D.C. Tumor collection protocols were approved by the respective IRB committees. The tumor samples were collected immediately after the surgery and frozen in liquid nitrogen. The ER status of the tumors collected from Howard University hospital and Providence hospital was obtained from the Tumor Registries of the respective hospitals. It was determined immunohistochemically by Oncotech laboratories using a monoclonal antibody against the NH_2-terminal domain (A/B region) of the receptor. A total of 25 ER^+ and 6 ER^- tumors derived from African American patients and 20 samples from Caucasian patients (obtained from the San Antonio Breast Tumor bank) were analyzed in the current study.

RNA isolation

Total RNA from African American patient tumors was isolated, using Trizol reagent and the manufacturer's protocol. Briefly, the tumors were first ground to a powder, using a cold, sterile pestle

and mortar, in the presence of liquid nitrogen. The pulverized tumor powder (50–100 mg) was suspended in 1 ml Trizol reagent, homogenized with a hand-held homogenizer and incubated at room temperature for 5 min to permit the dissociation of nucleoprotein complexes. Then, 0.2 ml chloroform was added to the above, shaken vigorously and centrifuged at 4 °C for 15 min at 12 000g to collect the supernatant. Total RNA was precipitated from the supernatant by adding 0.5 ml isopropanol. The precipitate was washed twice with 75% ethanol, dried briefly and dissolved in diethyl-pyrocarbonate-treated water. The RNA samples from ER^+ breast tumors of Caucasian patients were similarly isolated. The total RNA from the ER^+ MCF-7 cell line and three ER^- cell lines, LCC6, MDA-MB-235 and MDA-MB-435, were also isolated, using Trizol reagent as described above with a minor change. The cells were suspended by pipetting up and down in Trizol reagent instead of by homogenization. This method yielded about 30 µg total RNA/100 mg tumor tissue and 0.8–0.1 mg/10^7 tissue-culture cells. The integrity of the isolated RNA was verified by electrophoresis in 1.5% agarose gels in TRIS/acetate/EDTA buffer and the concentration was determined by measuring the absorbance at 260 nm. Only those RNA samples that demonstrated intact RNA bands revealed by ethidium bromide staining were chosen for the current study. The integrity of the RNA was further confirmed by RT-PCR amplification of glyceraldehyde-3 phosphate dehydrogenase transcripts.

Reverse transcription and PCR

The isolated RNA was reverse-transcribed to cDNA, using Maloney murine leukemia virus reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 µg total RNA, 2.5 units reverse transcriptase, 1 mM each dNTP, 2.5 µM random hexamers, 1 U RNase inhibitor, 5 mM $MgCl_2$ and 1× PCR buffer in a total volume of 20 µl. For reverse transcription of the RNA, the reaction tubes were first left at room temperature for 10 min, and then incubated at 42 °C for 15 min, 99 °C for 5 min and finally 5 °C for 5 min. For ER cDNA amplification, various primer sequences located in exons 1, 4, 6, 7 and 8, as listed in the text, were used. The positions of the primers are also shown schematically in Fig. 1. The sequence and locations of all the primers described in the present study are based on the full-length ER cDNA sequence published by Green and others (1986). The polymerase chain reactions were performed in an automatic thermal cycler (MJ Research) as described previously (Poda et al. 1998) in a 12.5-µl reaction volume containing the cDNA reverse-transcribed from 125 ng total RNA, 1× PCR buffer, 1× Q solution (Qiagen), 200 µM each dNTP, 2 µM sense primer, 2µM anti-sense primer and 0.6 U Taq polymerase. The PCR conditions were initial denaturation for 1 min at 95 °C followed by 94 °C for 1 min, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C for 40 cycles and final extension for 10 min at 72 °C. The presence of glyceraldehyde-3 phosphate dehydrogenase in tumor samples was verified by using a sense primer, 5′AAGGCTGAGAACGGG-AAGCTTGTCATCA-AT3′ (position, exon 3, bp 241–270), and an anti-sense primer, 5′TTCCCGTCTAGCTCAGGGATGACC-TTGCCC3′ (position, exon 7, bp 740–711) (Ercolani et al. 1988), under the same PCR conditions as for ER. All of the primer pairs
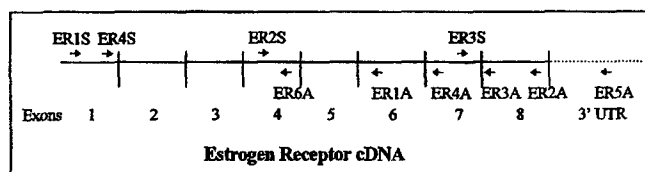
Fig. 1 Schematic representation of primers used in the current study for the amplification of various portions of estrogen receptor cDNA. The approximate primer locations in various exons are indicated with *arrows*
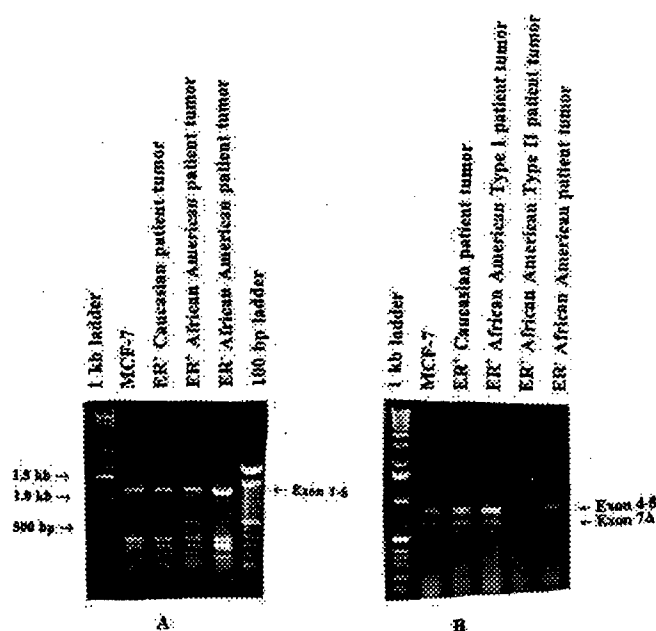
were first tested on the cDNA prepared from MCF-7 RNA and included as a positive control in all of the PCR amplifications. The RNA samples isolated from ER⁻ cell lines were used as negative controls.

Detection and sequence analysis of PCR products

To detect the PCR products, an aliquot of the reaction mixture (6 μl) was electrophoresed on 1% agarose gels and stained with ethidium bromide. To detect the variant ER products, the PCR were conducted in the presence of [α-^{32}P]dCTP at 0.5% of the total reaction volume and the radiolabelled PCR products were electrophoresed in a Bio-Rad vertical slab gel apparatus in 4.5% polyacrylamide gels, 90 mM TRIS/borate and 0.2 mM EDTA at 40 mA constant current for 3.5 h. The gels were dried in a Savant gel dryer and subjected to autoradiography using Kodak X-Omat AR film and DuPont NEN Lightning Plus intensifying screens at room temperature. In order to confirm the identity of the PCR-amplified ER products, they were electrophoresed in agarose gels and purified by QIAquick gel-extraction kit. The purified products were cloned into pCRII-TOPO vector and sequenced by the cycle sequencing method (Smith et al. 1986; McCombie et al. 1992) on an automated DNA sequencer (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, Md.).

Fig. 2A, B Amplification of estrogen receptor (*ER*) exons 1–6 and 4–8 in tumors from African American and Caucasian patients. The ER exons were amplified by reverse transcription-polymerase chain reaction (RT-PCR) and the products were detected by electrophoresis in 1% agarose gels and ethidium bromide staining. **A** The PCR products generated with primers ER1S and ER1A, which amplify exons 1–6 from MCF-7, a representative of 20 tumors from Caucasian patients, a representative of 23 ER⁺ tumors from African American patients, and a representative of 2 ER⁻ tumors from African American patients. **B** The wild-type and exon 7 deletion variant PCR products generated with ER2S and ER2A which amplify exons 4–8 from MCF-7, a representative of 20 tumors from Caucasian patients, a representative of 15 ER⁺ tumors from African American patients that gave positive products (type I), a representative of 8 ER⁺ tumors from African American patients that did not give positive products (type II) and a representative of 2 ER⁻ tumors from African Americans

Results

The ER mRNA profiles were studied by RT-PCR in tumors that were diagnosed for ER status by immunohistochemistry. We analyzed 25 ER⁺ and 6 ER⁻ malignant tumors from African American women and 20 ER⁺ malignant tumors from Caucasian patients. To detect ER transcripts, we employed primer pairs that could amplify exons 1–6, 1–4, and 4–8. When primers ER1S (5'CTCCACACCAAAGCATCTGGG3'; position, exon 1, bp 245–265) and ER1A (5'CCAGCAGCATGTCGAAGATC3'; position, exon 6, bp 1520–1501), which amplify exons 1–6 were used, 23 ER⁺ and 2 ER⁻ tumors from African American patients, and all 20 tumors from Caucasian patients gave a PCR product of the expected size (approx. 1.275 kb) corresponding to the wild-type ER. A PCR product profile from representative tumor samples and MCF-7 is shown in Fig. 2A. We did not detect any products in 2 of the ER⁺ tumors from African American patients either by ethidium bromide staining on agarose gels or as radiolabelled products on acrylamide gels. We analyzed exons 4–8 in all of the tumors using the primer pair ER2S (5'GCCCGCTCATGATCAAACGC3'; position, exon 4, bp 1113–1132) and ER24 (5'ATACTTTTGCAAGGAATGCGA3'; position, exon 8, bp 1978–1958). These primers amplified two products of sizes 866 bp and 682 bp in 15 ER⁺ tumors from African American patients, and all 20 tumors from Caucasian patients. The 866-bp and 682-bp products were identified as the wild-type and exon 7 deletion variant by sequence analysis. Two of the ER⁻ tumors from African American patients that were positive with primers ER1S and ER1A also amplified the 866 bp product but not the 682-bp product. A PCR product profile generated from MCF-7, a representative

of 20 tumors from Caucasian patients, a representative of 15 ER⁺ tumors from African patients that gave positive products (type I), a representative of 8 ER⁺ tumors from African American patients that did not give any products (type II) and a representative of 2 ER⁻ tumors from African American patients are shown in Fig. 2B. These results were repeatable in at least five different cDNA preparations from the tumor RNA and several PCR trials. Two ER⁺ tumors that did not give any products with ER1S and ER1A also did not yield any products with ER2S and ER2A detectable either by ethidium bromide staining on agarose gels or as radiolabelled products on acrylamide gels. The reasons for the absence of any transcripts in these 2 tumors are not known; however, it should be noted that immunohistochemistry showed very low levels (approx. 20 fM) of protein. These 2 tumors are probably falsely ER⁺ and were not analyzed further. Thus it appears that only 15 of the 25 ER⁺ tumors from African American patients showed full-length wild-type sequences. This was unexpected. We hypothesized that the 8 ER⁺ type II tumors that did not amplify with ER2S and ER2A may have

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had alterations and/truncations in exon 8. Therefore, we next examined these tumors for potential alterations and/or truncations in exon 8 with another antisense primer, ER3A (5'GCACTTCATGCTGTACAGATG-C3'; position, exon 8, bp 1822–1801) in exon 8 upstream of ER2A together with the sense primer ER2S. The results are shown in Fig. 3. The PCR products generated from MCF-7, a tumor from a Caucasian patient and one from a type I African American patient were also included as positive controls. Of the 8 type II tumors from African American patients, 2 gave a product that was slightly higher than the expected 710-bp wild-type band but did not amplify the exón 7 deletion product (type IIA). Two of the type II tumors gave the expected wild-type and exon 7 deletion products of 710 bp and 526 bp respectively (type IIB). One tumor did not amplify any product (type IIC) and 3 tumors amplified only the expected wild-type ER band but not the exon 7 deletion product (type IID). A PCR product profile from a representative of each of types IIA, -B, -C, and -D is shown in Fig. 3. The gel is overloaded to show the absence of the naturally occurring exon 7Δ variant in type IID tumors.

To test the possibility that a type IIC tumor may have the whole exon 8 truncation, we designed another anti-sense primer, ER4A (5'GTCCTTCTCTTCCAGA-GAC3'; position, exon 7, bp 1651–1633) in exon 7 and tested this together with ER2S. The results are shown in Fig. 4. The PCR products from MCF-7, a Caucasian patient's tumor and a type I tumor from an African American patient are included as positive controls. The type IIC tumor amplified an expected 538-bp wild-type product and an additional 400-bp product, which was identified as exon 5Δ. These results indicated the deletion of all of exon 8 in this tumor.

All of the above 8 type II tumors from African American patients that did not amplify the expected PCR products with ER2S and ER2A were further examined for potential alterations using a sense primer in exon 7, ER3S, 5'CAGGCCTGACCCTGCAGCAGC3' (position, bp 1710–1730) and an anti-sense primer in the non-coding region, ER5A, 5'TCTCCAAGTCACCAA-TTAAAGG3' (position, bp 2474–2454). These two primers amplified an expected 764-bp product from MCF-7 and all type I tumors from African American patients but did not yield a corresponding band in any of the 8 type II tumors (data not shown).

Since the primer pair ER2S and ER4A co-amplified exon 5Δ along with wild-type sequences, they were used
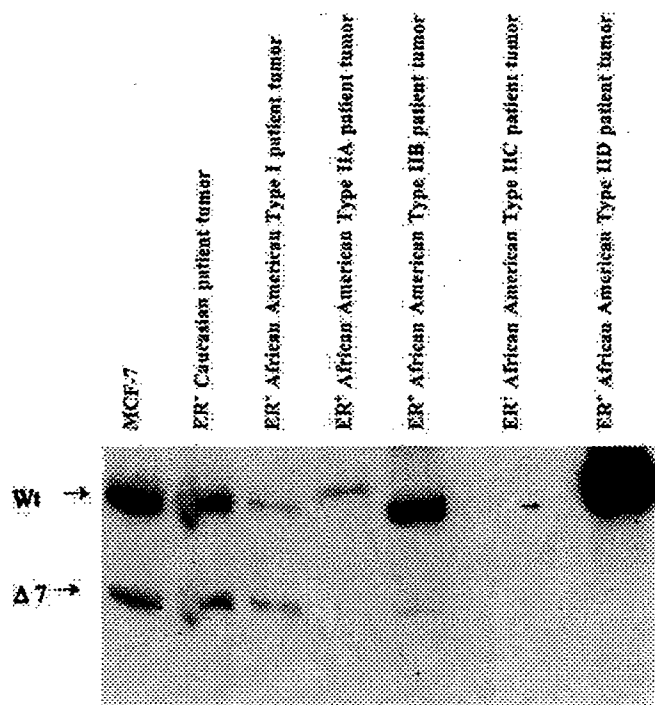
Fig. 3 Testing of ER+ type II tumors from African American patients for possible truncations/alterations in exon 8. All the tumors that did not give any PCR products with ER2S and ER2A were tested with ER2S and an anti-sense primer, ER3A, upstream of ER2A under the PCR conditions described in Materials and methods. The PCR were conducted in the presence of [α-^{32}P]dCTP and the products detected by autoradiography. The PCR products from MCF-7, a representative from 20 tumors from Caucasian patients, and a representative from 15 type I tumors from African American patients are shown as positive controls. A representative of type IIA, type IIB, type IIC and type IID tumor products are shown
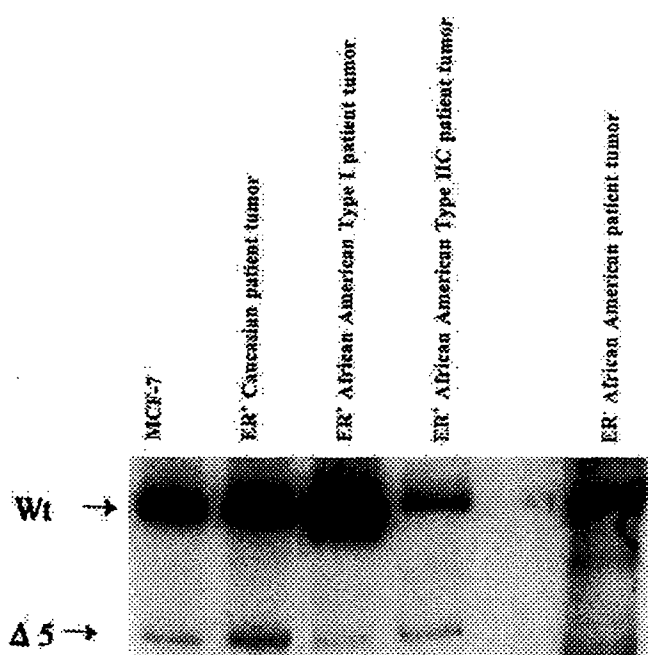
Fig. 4 Testing of type IIC ER+ tumor for a possible complete deletion of exon 8. One of the ER+ tumors from an African American patient, which was positive for exons 1–6 but did not amplify any product between exons 4 and 8, was tested with primers that amplify exons 4–7. The amplifications were conducted in the presence of [α-^{32}P]dCTP and the product(s) were analyzed by autoradiography as described. The PCR products from MCF-7, a representative tumor from a Caucasian patient and a representative type I tumor from an African American patient are shown as positive controls. The PCR product(s) from a representative ER⁻ tumor from an African American patient, which was positive for the full-length wild-type sequence, is also shown along with those of the type IIC tumor
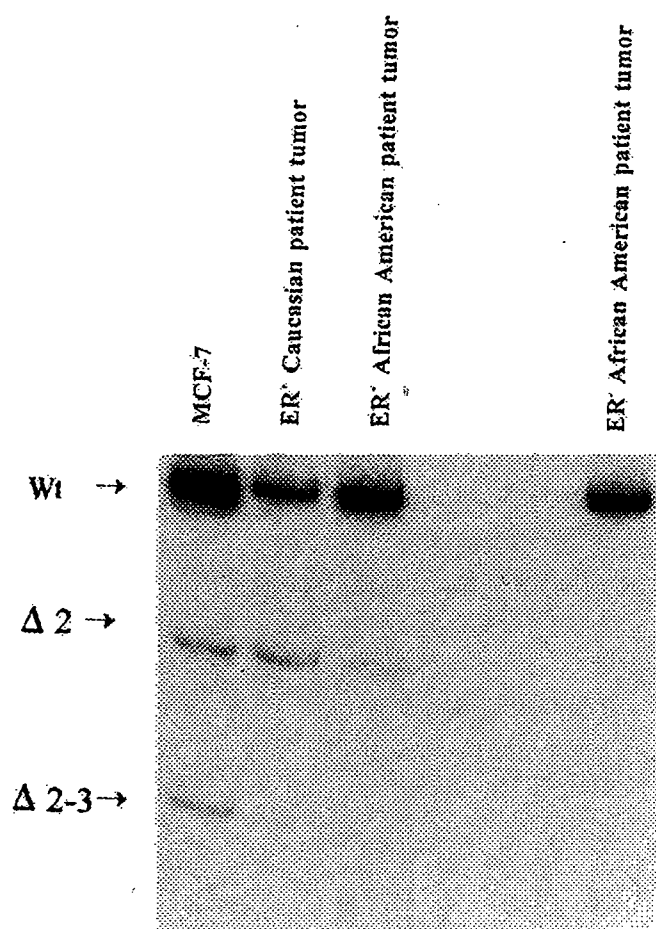
Fig. 5 Amplification of exons 1–4 to identify exon deletion variants in this region. To analyze the ER splice variants between exons 1 and 4, all tumor cDNA were subjected to PCR amplification in the presence of [α-^{32}P]dCTP with primers ER3S and ER5A and the products were identified by autoradiography. The PCR products from MCF-7, a representative tumor from a Caucasian patient, a representative ER$^+$ tumor from an African American and a representative ER$^-$ tumor from an African American are shown

Table 1 Estrogen receptor transcript profiles in breast tumors from Caucasian ($n = 20$) and African American women ($n = 25$)

No.	Transcript	Number of tumors expressing the transcript	
		Caucasian	African American
1	Full-length	20	15
2	Exon 8 truncated	0	8
3	Exon 7Δ	20	17
4	Exon 5Δ	2	23
5	Exon 2Δ	20	19
6	Exon 2–3Δ	20	22
7	No transcript	0	2
8	Complete exon 8 truncation	0	1

The results from representative tumors are presented in Fig. 5. All of the tumors from Caucasian patients and 19 ER$^+$ tumors from African American patients showed the presence of both exon 2Δ and exons 2–3Δ transcripts. An additional 3 ER$^+$ tumors from African American patients were positive for the exons 2–3Δ variant only. We did not observe any transcripts in any of the three ER$^-$ cell lines tested with any of the primer pairs. Table 1 summarizes the expression of various transcripts in tumors from Caucasian and African American patients. Two of the ER$^-$ tumors that showed the presence of the full-length wild-type sequence did not show the presence of either 2Δ or exons 2–3Δ. A PCR product from 1 of the 2 ER$^-$ tumors is shown in Fig. 5. Thus it appears that the ER$^-$ tumors that generated full-length wild-type PCR products did not express either exon 5Δ, exon 7Δ, exon 2Δ or exon 2–3Δ variants.

Discussion

We investigated the profiles of ER mRNA by RT-PCR in the tumors of African American women that are in general described as poorly differentiated and aggressive. We thought that the variations in the ER structure may, in part, contribute to the above characteristics. The results presented in this report clearly show that a considerable number of tumors contained mRNA that appear to have alterations/truncations in the major portion of exon 8. One of the 25 ER$^+$ tumors had exon 8 completely deleted. These are the first observations showing alterations/truncations in ER exon 8.

Several studies have reported the presence of ER mRNA that have truncations in exons 2, 3 and 6 in single breast tumors. The majority of the truncated transcripts contain the entire sequences of at least two of the 5' ER exons, and then diverge into ER-unrelated sequences (Dotzlaw et al. 1992). The role of one of the truncated variants, namely clone 4, which consists of exons 1 and 2 of the wild-type ER mRNA followed by unrelated sequences, has been evaluated. It was found that the clone 4 level was significantly higher in breast tumors than in the normal mammary gland (Leygue

to screen all of the tumors for the presence of 5Δ. We observed the presence of 5Δ in two tumors from the Caucasian patients, and in 23 ER$^+$ tumors from African American patients. However, it was absent in 2 of the ER$^-$ tumors that showed the presence of full-length sequences. A representative ER$^-$ tumor is shown in Fig. 4. A minor band was visible, which was larger than the exon 5 deletion product but was not characterized because of its very low abundance.

To detect the exon deletion variants in the exon 1–4 region, we designed another set of primers: a sense primer, ER4S (5'TGCCCTACTACCTGGAGAACG3'; position, exon 1, bp 615–635) downstream of ER1S and an anti-sense primer, ER6A, (5'GGTCAGTAAGCC-CATCATCG3'; position, exon 4, bp 1272–1254) in exon 4, and screened all of the tumors for the presence of variant transcripts. The primers ER4S and ER6A amplified three PCR products of 658 bp, 487 bp and 370 bp, which were identified as the wild-type, exon 2Δ and exon 2–3Δ transcripts respectively, by sequence analysis.

et al. 1996a). The relative level of this truncated variant was also found to be significantly elevated in breast tumors with characteristics of poor prognosis and endocrine resistance (Murphy et al. 1995). These reports suggest that the truncated transcripts play a role in developing hormone resistance and influence the disease outcome.

The truncations we have observed in the breast tumors of African American women appear to be different from the above-reported truncations in that the mRNA appear to have intact wild-type exons 1–7 but only a part of exon 8. Since we could not obtain any product with primers that amplify between exon 7 and part of the non-coding region in any of these tumors, it is possible that the non-coding sequence is also modified. Owing to the limited amount of tumor RNA, further investigation by Northern blotting could not be undertaken to characterize this modification. Since the exon 8 sequences encode a portion of domain E and the complete F domain, which controls ligand binding, ligand-dependent dimerization, ligand-dependent transactivating and transcriptional responses to estrogens and anti-estrogens, alterations/truncations in this exon will considerably alter the ligand binding properties of the receptor. Another distinguishing characteristic observed in the tumors from African American patients is that the majority of those that showed alterations/truncations in exon 8 did not express the naturally occurring, more abundant exon 7Δ. Although, in vitro studies have shown that exon 7Δ has a negative dominant effect on the wild-type receptor (Fuqua et al. 1992), it has been postulated that the variants, including the exon 7Δ, that are expressed in normal tissues play a role in the estrogen-induced signal-transduction pathway. The significance of the absence of the exon 7Δ is not known, but it may be relevant that this absence was observed only in tumors that did not demonstrate full-length transcripts. Our results also show that 2 of the truncated tumors that did not express the more abundant exon 7Δ variant had lower mobility than the expected wild-type product on polyacrylamide gels. This lower mobility may be due to base insertions. Because of the very limited amount of RNA from those tumors, the PCR products could not be cloned and sequenced to identify the site and nature of the modifications. However, since they generated PCR products of the expected size with primers that amplify exons 1–6, it is possible that the modifications could be either in exon 7 or in the untruncated portion of exon 8.

The presence of variants with deletions between exons 1 and 6 was also investigated using primers that amplify exons 1–4 and exons 4–7. Our study indicates that the majority of tumors co-expressed exon 2Δ, exons 2–3Δ, and exon 5Δ transcripts. The occurrence of the exons 2–3Δ in both normal and cancer tissues has been reported by Leygue and others (Fuqua et al. 1992; Leygue et al. 1996b). In a study of 100 tumors, they reported the presence of this multiple-deletion variant in less than 10% of the samples. In our study we found that all but 2 tumors expressed this variant. The exon 2Δ variant was detected in 9 out of 15 tumors with primers ER3S and ER5A. The exon 5Δ variant transcripts were detected in 23 tumors from ER$^+$ African American patients. These results show the co-expression of several exon deletion transcripts in the tumors examined.

Finally, very interesting observations were made with respect to tumors identified immunohistochemically as ER$^-$. We found that 2 of 6 tumors studied showed full-length ER mRNA sequences. We did not observe any products from ER$^-$ cell lines MDA-MB-435, MDA-MB-235 or LCC6 with any of the primer pairs tested. Unexpectedly, the above tumors did not express either exon 7Δ, exon 2Δ, exons 2–3Δ or exon 5Δ transcripts. The physiological significance of these observations is not known. The presence of wild-type full-length sequences in these tumors suggests two possibilities: (i) that the ER mRNA are not translated in these tissues or (ii) that the amounts of protein are undetectable by immunohistochemistry. It is not known whether the absence of immunohistochemically detectable protein in these tumors is related to the absence of variant transcript molecules. It is possible that the lower frequency of ER$^+$ tumors reported in African American patients may be due to the presence of altered receptors, which are not translated into immunoreactive ER protein.

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Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach

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Abstract

Estrogen receptor (ER) alpha splice variant transcript profiles were analyzed by RT PCR in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6, and three ER positive malignant breast tumors using targeted primers which specifically anneal to the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that largest possible transcripts were amplified between exons 1 and 8. The results described here show that each splice specific primer amplified not only the single exon deleted transcript but also a number of related transcripts that have deletions in various combinations of exons. The exon 2Δ specific primer amplified five transcripts that have deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4–5, and exons 2 and 4–6. The exon 3Δ specific primer amplified two transcripts that have deletions in exon 3, and exons 3 and 7. The exon 2–3Δ specific primer amplified three products that have deletions in exons 2–3, exons 2–3 and 7 and exons 2–3, 5 and 7. The exon 4Δ specific primer amplified two products that have deletions in exon 4, and exons 4 and 7. The exon 5Δ specific primer amplified three transcripts, that have deletions in exon 5, exons 5 and 2, and exons 5, and 2–3. The 6Δ specific primer amplified only one transcript that has a deletion in exon 6. The 7Δ specific primer amplified four transcripts, that have deletions in exon 7, exons 7 and 4, exons 7 and 3–4, and exons 7 and 3–5. None of the above splice specific primers amplified the wild type ER sequences. The six ER positive cell lines differed in the patterns of the variant transcripts and among the three ER negative cell lines analyzed, only MDA-MB-435 showed the presence of exon 2Δ and exon 4Δ transcripts. Analyses in the tumor samples indicated that the above transcripts are extensively modified. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: ER alpha splice variants; Splice targeted primers; Sequential exon deletions; Breast cancer cell lines and tumors; Distant exon deletions

1. Introduction

The ER alpha mRNA undergoes alternate splicing, generating transcripts containing single, double or multiple exon deletions. The presence of ERα transcripts with deletions in exons 2-, 3-, 2–3, 2–5, 4-, 5-, 6- and 7 has been described in breast cancer cell lines and normal- and malignant breast tissue samples [1–4]. Although the exact function(s) of these splice variants is not established, it has been hypothesized that the splice variant mRNAs may result in proteins that differ in activity. These may differentially modulate the ER signalling pathway in normal tissues. Consequently, changes in the balance of these transcripts could perturb the ER signaling pathway and contrib-

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Nomenclature

ER	estrogen receptor		Exon Δ	exon deletion
PgR	progesterone receptor		AX	anti-sense
GAPDH	glyceraldehyde 3-phosphate dehydrogenase		SX	sense

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ute to tumor progression. Several studies suggested that the expression of certain exon deletion transcripts is deregulated during breast tumorigenesis. It was shown that the exon 5 deletion transcript was significantly elevated in ER^- PgR^+ breast tumor tissues [5]. Elevated levels of exon 7 splice transcripts have also been reported in $ER^+/PR^-/pS2$ compared to ER^+/PgR^+ tumors [6]. It has been reported that expression of the exon 3-deleted mRNA is reduced in breast tumor tissue compared with normal tissue [7]. Differential expression of exon 5 and exon 7 deletion transcripts also seem to influence the estrogen responsiveness in breast cancer cell lines [8]. All these reports suggest that expression of some ER variants is altered in human breast tumors and may contribute to tumorigenesis, tumor progression and response to hormones. Therefore, it is important to qualitatively and quantitatively investigate the levels and pattern of ER splice variant expression between normal and neoplastic tissues, and amongst groups of tumors with different characteristics. Yet, there are no specific methods available which can precisely detect and quantify the alternatively spliced ER molecules.

Conventionally, the ER exon deletion variant transcripts are characterized by co-amplification with the wild type sequences using reverse transcription polymerase chain reaction (RT-PCR) approaches which by virtue of specific primer design are focussed on small regions of the known wild type mRNA. However, there are several practical limitations to this approach. Firstly, the threshold of detection — since the wild type transcripts are present in large excess to alternatively spliced molecules, a competitive amplification occurs amongst the wild type and all the alternatively spliced transcripts. Detection of products corresponding to alternatively spliced molecules depends upon the relative expression levels of their mRNA species within the sample. Thus, spliced transcripts expressed at low levels may fall below the threshold of detection. Secondly, this approach cannot distinguish those mRNAs with multiple deletions in distant exons. For example, an ER transcript which has deletions in exons 2 and 7 cannot be distinguished from transcripts having single deletions in exon 2 or exon 7 by this method, and finally transcripts with similar sized deletions cannot be distinguished by gel exclusion chromatography.

To circumvent all the above described limitations,

we have developed a new approach to characterize the alternatively spliced molecules. This involves the targeted amplification of the alternatively spliced molecules as separate gene populations without co-amplification of wild type molecules using specific primers designed for the alternative splice junctions [9]. In the current study, we analyzed the ER single, double, and multiple exon deletion variant transcripts in breast cancer cell lines and tumors by RT PCR using the splice targeted primers. We show here that each splice specific primer amplifies not only the single exon deleted transcript but also a number of related transcripts with deletions in various combinations of exons. Our results also show that several alternatively spliced molecules are either missing or extensively modified in tumor samples.

2. Materials and methods

AmpliTaq PCR core kits and QIAquick gel extraction kits were obtained from QIAGEN, Santa Clara, CA. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. Reverse transcriptase kits were purchased from Applied Biosystems. The pCR™2.1-TOPO cloning vector was obtained from Invitrogen. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, MD. The total RNA samples from breast cancer cell lines and tumors were prepared using Trizol reagent (Gibco-BRL). The integrity of all the RNA preparations was confirmed by electrophoresis and ethidium bromide staining and amplification of the constitutively expressed gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH). The ER status of all the tumors used in the current study was determined immunohistochemically by Oncotech laboratories using monoclonal antibodies against the NH_2 terminal (A/B region) of the receptor. The six tumors used were ER positive by the above immunohistochemical method.

2.1. Targeted primers for the amplification of single, double and multiple exon deletion variant cDNAs of ER

We have previously shown that the primers targeted at the alternate splice junctions that have a minimum of three out of four unique bases at the extreme 3' end

will specifically amplify the spliced junction without amplifying the flanking wild type exons and in order to design such a primer, the overhang sequences can extend up to eight bases past the splice junction [9]. The splice specific primers used in the current study were designed based on these principles. The splice specific primers used for amplifying 2Δ, 3Δ, 2-3Δ, 4Δ, 5Δ, 6Δ, and 7Δ were ER SX1/3, 5' CGCCGGCATTC-TACAG 1/3 GACAT 3' (positions, exon 1, bp 669–684, and exon 3, bp 876–880), ER SX2/4, 5' AAGA-GAAGTATTCAAG 2/4 GGATA 3' (positions, exon 2, bp 860–875 and exon 4, bp 993–997), ER SX1/4, 5' GCCGGCATTCTACAG 1/4 GGATAC 3' (positions, exon 1, bp 670–684 and exon 4, bp 993–998), ER SX3/5, 5' GTGGGAATGATGAAAGGTG 3/5 GCTTT 3' (positions, exon 3, bp 974–992 and exon 5, bp 1329–1333), ER AX4/6, 5' ATTTTCCCTGGTTC 6/4 CTGGCAC 3' (positions, exon 6, bp 1481–1468 and exon 4, bp 1328–1322), ER AX 5/7, 5' CAGAAATGTGTACACTC 7/5 CTGT 3' (positions, exon 7, bp 1618–1603 and exon 5, bp 1468–1465) and ER AX6/8, 5' CTCCATGCCTTTGTTA 8/6 CAGAA 3' (positions, exon 8, bp 1801–1786 and exon 6, bp 1601–1597), respectively. The partner primer for 2Δ, 3Δ, 2-3Δ, and 4Δ splice specific primers was ERA, 5' GCACTTCATGCTGTACAGATGC 3' (position, exon 8, bp 1822–1801) and for 5Δ, 6Δ, and 7Δ primers was ERS, 5' TGCCCTACTACCTGGAGAACG 3' (position, exon 1, bp 615–635). The sequence and locations of all the primers described here are based on the full length ER cDNA sequence published by Green et al. [10].

2.2. Reverse transcription and PCR

The total RNA was reverse transcribed to cDNA using Maloney Murine Leukemia Virus reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 µg of total RNA, 2.5 units of MuLV reverse transcriptase, 1 mM each of dNTPs, 2.5 µM random hexamers, 20 U of RNAse inhibitor, 5 mM MgCl₂ and 1 × PCR buffer in a total volume of 20 µl. To reverse transcribe the RNA, the reaction tubes were first left at room temperature for 10 min, followed by incubations at 42°C for 15 min, 99°C for 5 min and finally 5°C for 5 min. The polymerase chain reactions were performed in an automatic thermal cycler (MJ Research) as described previously [11] in a 25 µl reaction volume containing the cDNA reverse transcribed from 250 ng of total RNA, 1 × PCR buffer, 1 × Q solution, 200 µM each of dNTPs, 2 µM each of sense and anti-sense primers and 0.6 U of Taq polymerase. The GAPDH was amplified using a sense primer, 5' AAGGCTGA-GAACGGGAAGCTTGTCATCAAT 3' (position, exon 3, bp 241–270), an anti- sense primer, 5'

TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3' (position, exon 7, bp 740–711) [12] and cDNAs prepared from reverse transcription of 25 ng of total RNA. To amplify the exon deletion variant cDNAs in the tumor samples, PCRs were performed using cDNAs prepared from reverse transcription of 500–750 ng of total RNA. The PCR conditions were initial denaturation for 1 min at 95°C followed by 94°C for 1 min, annealing for 1 min at the specified temperature depending on the primer pair used, extension for 2 min at 72°C for 40 cycles and final extension for 10 min at 72°C. The annealing temperature for 2Δ, 2-3Δ, 4Δ and 6Δ specific primers was at 61°C, for 3Δ and 7Δ primers at 55°C and for 5Δ specific primer at 65°C.

2.3. Detection and sequence analysis of PCR products

To detect the PCR amplified ER splice variant products from cell lines, an aliquot (4–7 µl) was electrophoresed in 1% agarose gels in Tris-acetate EDTA buffer and detected by ethidium bromide staining. To detect the PCR products of GAPDH, 1 µl was electrophoresed and the ER splice variant product amplified from tumor samples, 12–25 µl of the products were analyzed on the gel. To determine the identity of the PCR amplified ER splice variant products, they were electrophoresed in 1.2% agarose gels and purified individually using the QIAquick gel extraction kit. The purified products were cloned into pCR*2.1-TOPO vector and sequenced by cycle sequencing method on an automated DNA sequencer (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

3. Results

We analyzed the ER single, double, and multiple exon deletion transcripts by RT PCR using primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2-3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that the largest possible transcripts were amplified between the exons 1 and 8. This permitted the amplification of not only the single exon deletion transcripts but also those with multiple deletions in distant exons. The PCR analyses were carried out in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, and LCC9 and three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6. Three ER positive breast tumor samples were also included to test the applicability of splice targeted primer approach in analyzing the above transcripts in clinical samples. The results described here on the analysis of various alternatively spliced ER transcripts were repeated in 20 experimen-

tal trials with cell lines and three trials with tumor samples.

3.1. Analysis of exon 2Δ transcripts

The exon 2Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 1. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. The ER positive cell lines, MCF-7, ZR-75, LCC1, LCC2, and LCC9, amplified three major bands of sizes about 960, 780, and 640 bp. The cell line T47D did not amplify the 960 band, instead it amplified two products which are higher than 960 bp. All six ER positive cell lines amplified several minor bands ranging from 480–330 bp. Unexpectedly, one of the three ER negative cell lines tested, MDA-MB-435, also amplified 960, 640 and 480 bp bands and three additional bands that showed lower mobility than the 960 bp band. Tumor 3 did not amplify any product. Tumor 2 amplified minor bands at 640 and 480 bp and tumor 1 amplified only the 480 bp one as a minor band. To determine the identify of the above products, the PCR products from LCC1 cells were cloned and sequenced. The 960, 780, 640, 480 and 330 bp products were identified as ER transcripts with deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4–5, and exons 2 and 4–6, respectively (Fig. 1B). It was

also found that the exons 2Δ and 4–6Δ product had 20 bps missing in exon 7. Fig. 1(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.2. Analysis of exon 3Δ transcripts

The exon 3Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 2. Lanes M1 contain Gibco-BRL 100 bp ladders. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two products of sizes about 845 and 661 bp. The ER negative cell lines and two of the tumors in the study did not amplify these two bands. Only one of the three tumors (Tumor 5) amplified the 845 bp but not 661 bp product. To determine the identity of the above products, the PCR products from LCC1 cell line were cloned and sequenced. The 845 and 661 bp products were identified as ER transcripts that have deletions in exon 3, and exons 3 and 7, respectively (Fig. 2B). Fig. 2(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.3. Analysis of exons 2–3Δ transcripts

The PCR product profiles of exon 2–3Δ transcripts

ER 2Δ SPLICE VARIANTS
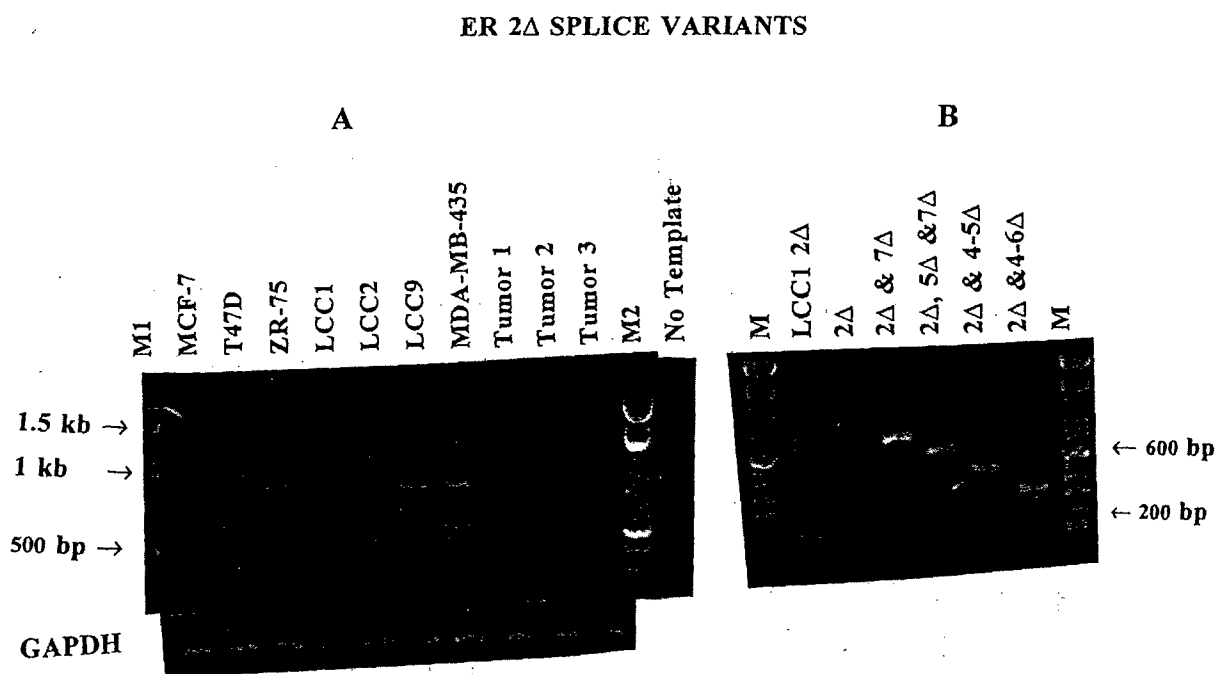

Fig. 1. Analysis of ER exon 2Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2Δ specific primer. The ER exon 2Δ transcripts were analyzed using the specific sense primer, ER SX1/3, and an anti-sense primer ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435, and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lanes M have 100 bp ladders.
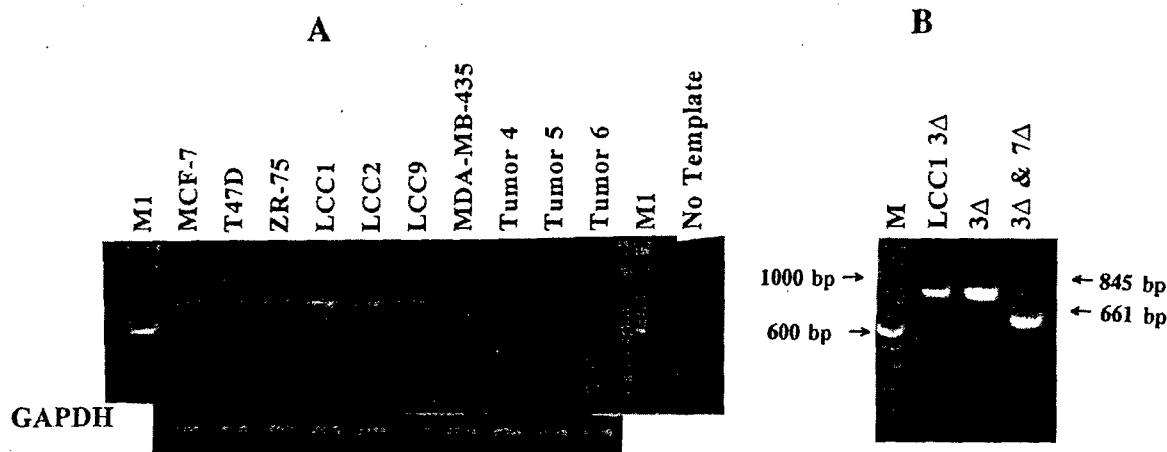
ER 3Δ SPLICE VARIANTS

Fig. 2. Analysis of ER exon 3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 3Δ specific primer. The ER exon 3Δ transcripts were analyzed using ER SX2/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

in seven cell lines and three tumors are shown in Fig. 3(A). The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. All the six ER positive cell lines amplified three products with approximate sizes of 840, 660 and 520 bp. Two minor bands between 840 and 660 bp are also seen. One of

the three ER negative cell line, MDA-MB-435, generated a minor product slightly bigger than the 840 bp product. To determine the identities of 840, 660 and 520 bp products, the PCR products from LCC1 were cloned and sequenced. The 840, 660, and 520 bp products were identified as ER transcripts with deletions
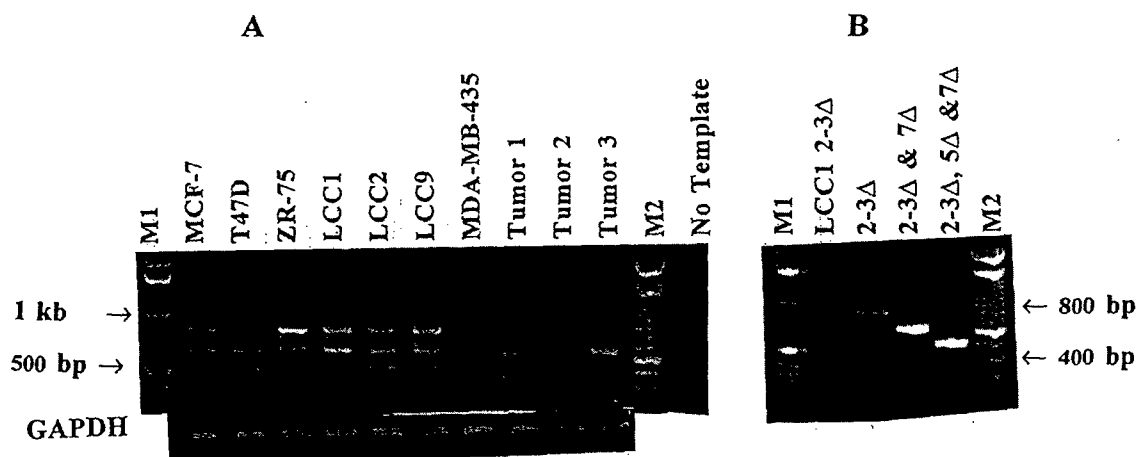
ER 2-3Δ SPLICE VARIANTS

Fig. 3. Analysis of exons 2–3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2–3Δ specific primer. The exon 2–3Δ transcripts were analyzed using ER SX1/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. In both A and B panels, lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis.

in exons 2–3, exons 2–3 and 7, and exons 2–3, 5 and 7, respectively (Fig. 3B). Tumor 1 generated three bands of which two corresponded to exons 2–3Δ, and exons 2–3Δ and 7Δ. The third band showed slightly higher mobility than the exons 2–3Δ, 5Δ and 7Δ product. Tumor 2 amplified two bands of approximate sizes 700 and 550 bp, which are slightly higher than the exons 2–3Δ and 7Δ, and exons 2–3Δ, 5Δ and 7Δ products. The third tumor generated only the exons 2–3Δ and 7Δ product. Fig. 3(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.4. Analysis of exon 4Δ transcripts

The exon 4Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 4. The lane M1 contains Gibco-BRL 100 bp ladder. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two products of sizes about 512, and 328 bp. One of the three ER negative cell lines, MDA-MB-435, also amplified faint bands of 512 and 328 bp. All three of the tumors tested amplified these two products. To identify the above products, the PCR products from LCC9 cell line were cloned and sequenced. The 512, and 328 bp products were identified as ER transcripts with deletions in exon 4, and exons 4 and 7, respectively (Fig. 4B). Fig. 4(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.5. Analysis of exon 5Δ transcripts

The profiles of exon 5Δ transcripts in seven cell lines and three tumors are shown in Fig. 5. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. All the ER positive breast cancer cell lines except MCF-7 amplified one major product and two minor products of approximate sizes, 730, 540 and 420 bp, respectively. The MCF-7 and all the three ER negative cell lines did not generate any products. To determine the identity of 730, 540 and 420 bp products, the PCR products from ZR-75 were cloned and sequenced. The 730-, 540- and 420 bp products were identified as ER transcripts having deletions in exon 5, exons 5 and 2, and exons 5 and 2–3, respectively (Fig. 5B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above three products and an additional product between exon 5Δ and the exons 5Δ and 2Δ products. Tumor 2 amplified one product between exon 5Δ and exons 5Δ and 2Δ products similar to tumor 1 and two products of approximate sizes 500 and 350 bp. Tumor 3 amplified only the 500 and 350 bp products. Neither tumor 2 nor 3 amplified the major single deletion product. Fig. 5(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.6. Analysis of exon 6Δ transcripts

The profiles of exon 6Δ transcripts in seven cell lines and three tumors are shown in Fig. 6. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders,

ER 4Δ SPLICE VARIANTS

Fig. 4. Analysis of ER exon 4Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 4Δ specific primer. The ER exon 4Δ transcripts were analyzed using ER SX3/5 and ERA. To determine the identity of various PCR products, the products from LCC9 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

ER 5Δ SPLICE VARIANTS

A   **B**

Fig. 5. Analysis of exon 5Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 5Δ specific primer. The exon 5Δ transcripts were analyzed using ER AX4/6 and a sense primer ERS. To determine the identity of various PCR products, the products from ZR-75 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the GiBco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

respectively. All ER positive breast cancer cell lines amplified one major product of approximate size 866 bp. It was identified as the transcript that has a deletion in exon 6 (Fig. 6B). None of the ER negative cell lines amplified any product. We could not detect any double or multiple deletion transcripts with 6Δ primer. The three tumors analyzed did not amplify any products (Fig. 6A). Fig. 6(A) also shows the expression

levels of GAPDH in the above cell lines and tumors and no template control.

3.7. Analysis of exon 7Δ transcripts

The profiles of exon 7Δ cDNAs in seven cell lines and three tumors are shown in Fig. 7. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders,
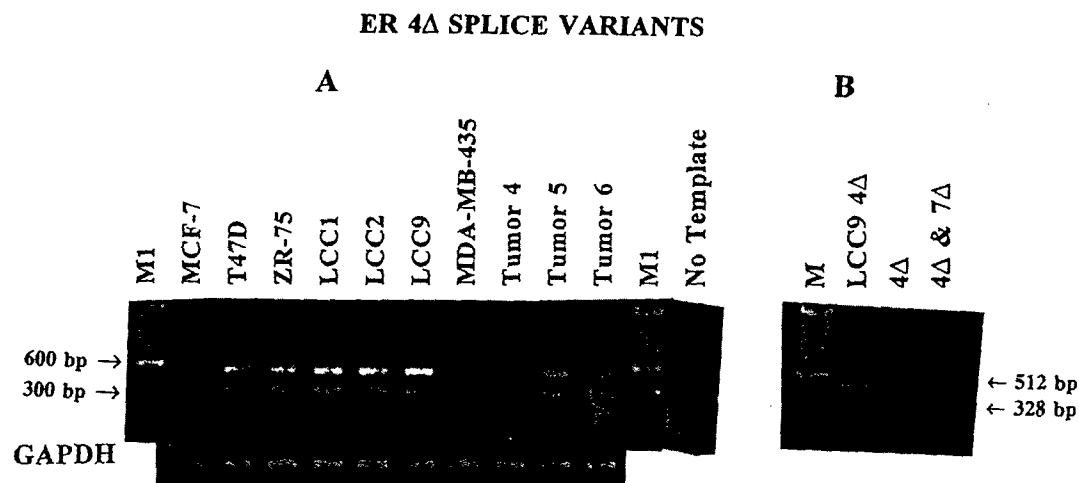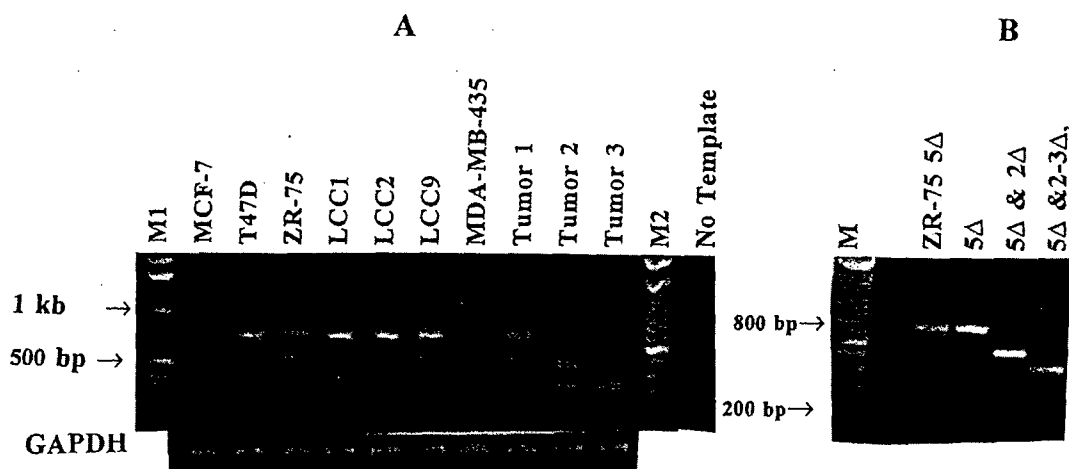
ER 6Δ SPLICE VARIANTS

A   **B**

Fig. 6. Analysis of exon 6Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 6Δ specific primer. The exon 6Δ transcripts were analyzed using ER AX5/7 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435, and the tumors 4, 5, and 6. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR product as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

respectively. All the six ER positive breast cancer cell lines generated a major 1 kb band and a minor band of approximately 665 bp. The cell line LCC2 generated an additional two minor bands of sizes 560 and 410 bp. The cell line LCC1 also generated 560 bp minor band and LCC9 generated the 410 bp minor band. In all these cell lines, several closely spaced minor bands were visualized between 1 kb and 665 bp products. To determine the identities of 1 kb, 665, 560 and 410 bp products, the PCR products from LCC1 were cloned and sequenced. They were identified as ER transcripts with deletions in exon 7, exons 7 and 4, exons 7 and 3–4, and exons 7 and 3–5, respectively (Fig. 7B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above four products, similar to LCC1 cell line. However, the exons 7Δ and 4Δ product is seen as a major band and the single deletion 1000 bp product as a minor band. Tumor 2 gave a similar profile to tumor 1, and tumor 3 did not amplify any product. Tumor 3 was previously shown not to have any exon 7Δ transcript when analyzed by co-amplification with wild type sequences between exon 4–8 [13]. Fig. 7(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

4. Discussion

In the current study we applied a novel approach to specifically amplify a particular category of alternatively spliced ER molecules, from a pool of other alternatively spliced and wild type ER genes, using primers which anneal to the spliced junctions. We used primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ transcripts. The results described above on the identities of various transcripts, amplified by the seven splice specific primers, are summarized in Table 1. Each splice specific primer amplified not only the single exon deleted transcript but also a number of related cDNAs that have deletions in various combinations of exons. None of the splice specific primers amplified the wild type ER sequences. The seven specific primers amplified a total of 20 transcripts, of which 14 had double or multiple exon deletions. Although single, a few double, and multiple deletion variants have been described, most of the double and multiple deletion transcripts described here were not previously reported.

Our results show that 10 of the 20 transcripts identified have exon 7 deletion, suggesting that this is the most frequently deleted exon. Examination of the products amplified by exon 2Δ, exon 3Δ, and exon 4Δ specific primers indicated a trend in the deletion of exons. In all these cases, the double deletion transcript identified had the deletion of exon 7 (Figs. 1B, 2B and 4B). A similar trend was seen for the exons 2–3Δ primer amplified products (Fig. 3B). These results suggest that initial deletion of a particular exon is mostly followed by the deletion of exon 7. Interestingly, the exon 7Δ specific primer recognized only one of the double deletion products, the exons 7Δ and 4Δ (Fig. 7B). This preferential amplification may be due
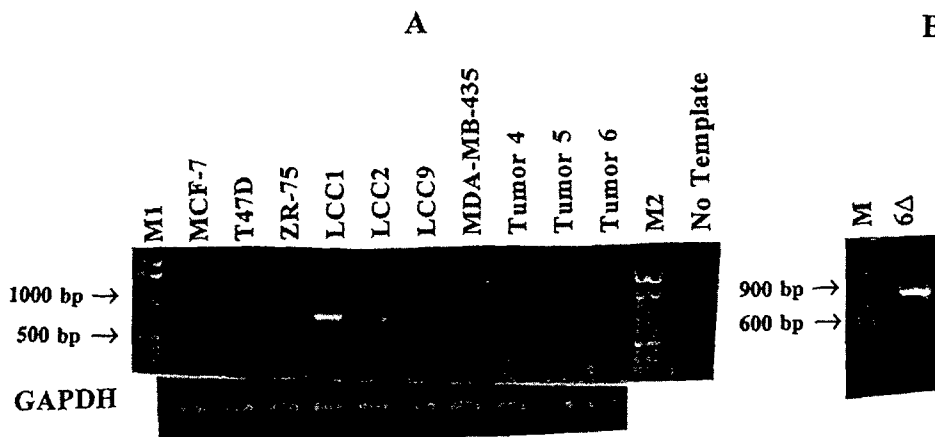
ER 7Δ SPLICE VARIANTS
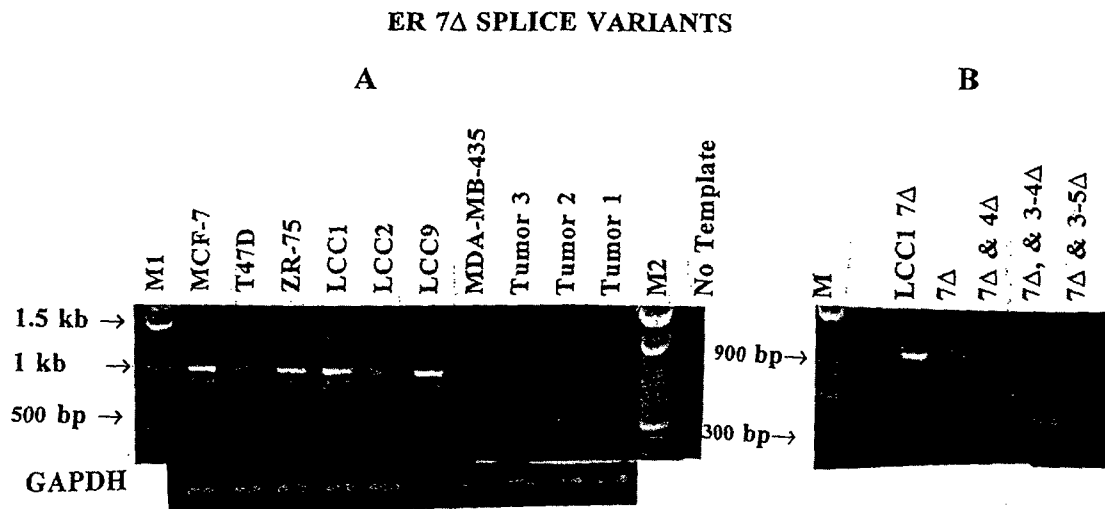
Fig. 7. Analysis of exon 7Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 7Δ specific primer. The exon 7Δ transcripts were analyzed using ER AX6/8 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

to competition among various transcripts. The detection of double deletion transcripts, the exons 5Δ and 7Δ, and exons 6Δ and 7Δ, was not possible in our studies because of the 5Δ and 6Δ specific primers design. The data presented here also show that the third largest cDNA amplified by 2Δ and exons 2–3Δ specific primers had the deletion of exon 5, suggesting that the third most common exon to be deleted in a transcript after the deletion of exon 7 is the exon 5. These observations also indicate that alternative splicing of the ER transcript takes place in a sequential manner, rather than at random. The 3Δ targeted primer did not amplify the triple deletion transcript, which lacked exons 3, 5, and 7 in our studies, probably due to its low abundance. The 4Δ primer did not amplify because of its unique design.

Among the seven targeted primers tested, only 2Δ and 7Δ primers amplified the transcripts with deletions in consecutive exons (Figs. 1B and 7B, respectively and the Table). The profile of these transcripts suggests that after the deletion of exon 2 in a transcript, if the second deletion is initiated at exon 4, the deletions seem to proceed up to exon 5 or 6. Similarly, after exon 7 deletion, if the second deletion is initiated at exon 3, the deletions seem to proceed up to exon 4 or 5. Examination of the other multiple deletion transcripts indicated that none of those had single exon 3Δ, instead, the deletion of exon 3 appears to be associated with either exon 2 or exon 4 deletion (Fig. 3B and 7B, respectively).

The results presented in Fig. (1)–(7) show some differences between estrogen dependent and independent ER positive cell lines in the patterns of variant transcripts. The LCC1, LCC2, and LCC9 are estrogen independent cell lines derived from the estrogen-sensitive parent cell line, MCF-7, after exposure to steroidal (ICI 182, 780)- or non-steroidal (Tamoxifen) anti-estrogens [14], [15]. These three cell lines did not show any differences in variant expression, suggesting that no ER remodeling is associated with either acquired Tamoxifen [14] or Tamoxifen and ICI 182,780 crossresistance [15]. In contrast, there seems to be some differences in ER variant expression associated with acquired estrogen-independence in these cells. For example, all three of the estrogen-independent cells contain the exons 7Δ, and 3–4Δ and exons 7Δ, and 3–5Δ transcripts. These are absent in the parental MCF-7 cells, and in the T47D and ZR-75 cells. Loss of exon 7 might be expected to affect ligand binding as might deletion of exon 5 and possibly exon 4. The entire hinge region would be lost in the 3–4Δ and 3–5Δ containing transcripts. Elimination of the ligand binding domain and part of the hinge region can produce transcriptionally active protein [16], overexpression of which could contribute to estrogen independence. While expression of the exons 7Δ, and 3–4Δ and exons 7Δ, and 3–5Δ transcripts is associated with acquired estrogen-independence, their function and whether significant amounts of these proteins are made, remain unclear. Another major difference observed is the absence of exon 5Δ, exons 5Δ and 2Δ and exons 5Δ, and 2–3Δ transcripts in the parental MCF-7 cells (Fig. 5A). It is possible that, these cells are estrogen dependent, in part, because of the absence of 5Δ transcript, which was reported to possess ligand independent transcriptional property. However, absence of 5Δ transcript alone may not determine the estrogen dependency because this transcript is detected in both T47D and ZR-75. It is possible that several splice variants, and their relative amounts to the wild type alpha receptor and the amounts of beta receptor in a given cell may influence estrogen dependency rather than a single transcript.

The exon deletion transcript analysis in tumor samples showed very interesting findings. In the cell lines, the most abundant product each specific primer amplified was the single deletion product and the second most abundant product was the double exon deleted transcript in the case of exon 2Δ, exon 3Δ, exon 4Δ, exon 5Δ and exon 7Δ. In the case of exons 2–3Δ specific primer, they are double and triple exon deleted transcripts. However, different primers gave different results in tumor samples. When three tumors were analyzed with exon 7Δ specific primer, two tumors showed the presence of four transcripts similar to the cell lines. However, the ratio of each transcript appears to be different compared to the cell lines. In the case of exon 2Δ transcripts, only two tumors

Table 1
Identities of twenty ER alpha spliced variants amplified by seven targeted primers

No.	Splice specific primer	cDNAs amplified
1.	ER SX1/3	2Δ, 2Δ & 7Δ, 2Δ, 5Δ & 7Δ, 2Δ & 4–5Δ and 2Δ & 4–6Δ
2.	ER SX2/4	3Δ and 3Δ & 7Δ
3.	ER SX1/4	2–3Δ, 2–3Δ & 7Δ and 2–3Δ, 5Δ & 7Δ
4.	ER SX3/5	4Δ and 4Δ & 7Δ
5.	ER AX4/6	5Δ, 5Δ & 2Δ, and 5Δ & 2–3Δ
6.	ER AX5/7	6Δ
7.	ER AX6/8	7Δ, 7Δ & 4Δ, 7Δ & 3–4Δ, 7Δ & 3–5Δ

showed the presence of minor bands and none of them amplified the single or double deletion products. When analyzed for the exons 2–3Δ containing transcripts, only one of the tumors generated 2–3Δ product, and the other two amplified the multiple deletion products, that appear to have other modifications, such as base pair insertions/deletions (Fig. 3A). Similar observations were made when analyzed for exon 5Δ transcripts (Fig. 5A). In summary, 5Δ and 2–3Δ transcripts are altered for base pair deletions and alterations, 2Δ, 3Δ and 6Δ transcripts are mostly absent, 7Δ transcript ratios are altered and 4Δ transcripts are unchanged in the tumor samples. These results suggest that the patterns and levels of ER variants undergo extensive alterations in tumor tissues.

The results presented in the current study clearly demonstrate the efficacy of the novel approach for analyzing the ER splice variant transcripts in the cell lines and tissue samples using targeted primers designed at alternate splice junctions. We believe that the new approach described here will be useful in: (1) delineating the functional roles of ER exon deletion variants in estrogen induced signal transduction processes, (2) analyzing the changes in the profiles of splice variants in the tumor tissues compared to normal tissues, (3) evaluating their role in tumorigenesis, tumor progression and loss of hormone dependency, (4) predicting prognosis and response to anti-hormone therapy, and finally (5) developing tissue specific synthetic estrogens and anti-estrogens.

Acknowledgements

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FREQUENCY OF ALTERNATIVELY SPLICED ESTROGEN RECEPTOR ALPHA mRNA EXPRESSION IS INCREASED IN MALIGNANT BREAST TISSUES

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Running Title. Alternatively spliced ERα isoform expression in breast tissues

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ABSTRACT

We previously identified twenty different alternatively spliced estrogen receptor alpha (ERα) mRNAs that have deletions in various combinations of exons in breast cancer cell lines using a novel 'Splice Targeted Approach' (Poola, Koduri, Chatra and Clarke, J. Steroid Biochemistry and Molecular Biology, 72, 249-258, 2000). In the current study, we compared the frequency of alternatively spliced ERα variant expression in 35 reduction mammoplasty and 38 breast cancer tissues with known ERα status using this highly specific 'Splice Targeted Approach'. A total of fifteen different alternatively spliced variants were identified that have deletions in various combinations of exons in normal as well as cancer tissues. However, not all fifteen variants were present in every tissue. The frequency and types of variants in normal and cancer tissues were significantly different. Majority of normal tissues expressed only single exon deletion variants with the exception of those in combination with exon 2Δ and exon 7Δ. Tumor tissues, on the other hand, showed increased frequency of multiple exon deletion mRNAs ($p < 0.019$). In addition, cancer tissues also showed increased frequency of all variants compared to normal tissues ($p < 0.044$). Specifically, we detected four different mRNAs that have exon 2 deletion: exon 2Δ, exons 2 & 4Δ, exons 2 & 5Δ, and exons 2, 4-5Δ in various combinations in both normal and cancer tissues. A large number of normal tissues expressed two transcripts, exon 2Δ and exons 2, 4-5Δ. The multiple exon deletion 2, 4-5Δ predominated in cancer tissues. Only the single exon 3 deletion variant, exon 3Δ, was detected in normal tissues. Cancer tissues showed the presence of a double exon deletion variant, exons 3 & 7Δ, in addition to exon 3Δ. A small fraction of normal tissues showed exons 2-3Δ mRNAs, whereas cancer tissues showed increased frequency of exons 2-3Δ expression in addition to a triple exon deletion variant, exons 2-3, & 5Δ. The expression of exon 4Δ or exons 4 & 7Δ or both was equivalent in normal and cancer tissues. Exon 5Δ transcripts were present at very low levels in both normal and tumor tissues since only a small sized transcript could be amplified. A small percentage of cancer tissues

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but not normal tissues showed exon 6Δ mRNA. The presence of single, double, triple and quadruple exon deletion mRNAs, exon 7Δ, exons 7 & 4Δ, exons 7, 3-4Δ, and exons 7, 3-5Δ respectively were detected in normal as well as cancer tissues. Each normal and cancer tissue had a distinct profile of ERα wild type and ERα splice variants. Heterogeneity in ER isoform profiles may result in variations in estrogen/anti-estrogen binding and activation/inactivation of estrogen-dependent genes, and therefore, may have implications in the risk of developing breast cancer, survival with the disease and response to anti-estrogen-, as well as other therapies. .

INTRODUCTION

A number of studies have reported the presence of alternatively spliced estrogen receptor alpha (ERα) mRNAs in both normal and malignant tissues (1-3). Although all known alternatively spliced ER proteins have not been characterized due to practical limitations in their detection, some of the translated protein products of both alpha and beta ERs have been found naturally in the breast tissues. For example, the predicted protein products of ERα exon 5Δ variant and a protein corresponding to transcript that has exon 6 and 7 duplication have been detected naturally (4, 5). A protein of M_r 60 kDa, corresponding to exon 7Δ, could be translated *in vitro* using mRNA from breast cancer cells (6). The presence of ERβ splice variant proteins in breast cancer cell lines and tumors have also been reported (7). These and the results of several other studies (8, 9) are consistent with the ability of ER variant mRNAs to be stably translated *in vivo* and therefore may have a functional role(s). *In vitro* functional analyses in yeast demonstrated that the spliced variants have distinct transcriptional properties compared to wild type ERα. For example, an exon 5Δ variant activates transcription in a constitutive manner in the absence of the hormone (10) and an exon 7Δ variant forms a heterodimer with the wild type receptor and exerts a dominant negative effect due to constitutive DNA binding activity in the absence of the hormone (11). *In vitro* transfection studies (12) with individual single exon deletion cDNAs in mammalian system suggested that the variant proteins mediate both positive and negative effects on estrogen-mediated gene transcription. It was also reported that breast cancer cell lines that have differential expression of splice variants respond differently to estrogens (13). All these reports suggest that the alternatively spliced ERs exhibit variable binding properties to estrogens/ anti-estrogens and transcriptional activation/inactivation of estrogen-responsive genes. Therefore, the presence of spliced variants may have important clinical implications in devising strategies for the prevention and treatment of breast cancer.

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breast cancer cell lines also indicated that the alternate splicing of ERα mRNA takes place in a sequential manner. The initial deletion of a particular exon seems to be mostly followed by the deletion of exon 7 and then exon 5. An exception to this trend seems to be after initial deletion of exon 2. We found that after an exon 2 deletion in a transcript, the second deletion could be either exon 4, or 5 or 7 or 4-5 or 4-6. Similarly, if exon 7 is deleted first in a transcript, it is mostly followed by the deletion of exon 3 and may proceed up to exon 4 or 5 (19). We also reported that estrogen independence is associated with increased levels of mRNAs that have multiple exon deletions in combination with exon 7 deletion (20). We tested the 'Splice Targeted Approach' in three breast tumor samples and found it to be applicable to analyze splice variants in biopsy samples (19).

In the current study, we compared the prevalence of alternatively spliced ERα mRNAs in thirty five reduction mammoplasty samples and thirty eight breast cancer tissues with known wild type ERα status using the 'Splice Targeted Approach'. Our results indicate that each normal/tumor tissue has a distinct profile of wild type- and splice variants of ERα. The heterogeneity in ER isoform profiles may result in variations in estrogen/anti-estrogen binding and activation/inactivation of estrogen-dependent genes, and therefore, may have implications in the risk of developing breast cancer, survival with the disease and response to anti-estrogen-, as well as other therapies.

MATERIALS AND METHODS

HotStartTaq PCR core kits and QIAquick gel extraction kits were from QIAGEN Inc., Santa Clara, CA. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. The pCR®2.1-TOPO cloning vector was obtained from Invitrogen. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, MD.

RNA extraction and cDNA synthesis. Frozen breast tissue was pulverized using a mortar and pestle and total RNA extracted with Trizol (Life Technologies, Paisley, UK) according to manufacturer's instructions. RNA from MCF-7 cell line was available from previous studies (19). RNA was used as a template for first strand cDNA synthesis as previously described (21). cDNAs were prepared from 35 reduction mammoplasty-tissues and 38 individual breast tumors that have been analyzed previously for ERα by either immunohistochemical, RT PCR or both methods as described (22-24). Tumors, classified by a Consultant Pathologist, were 15 node positive and 23 node negative, from patients of age 38-84 years (mean age 63). Four were grade I, 16 were grade II and 18 were grade III. Of the thirty eight tumor tissues included in the study, twenty nine were ERα- positive and nine were ERα-negative. All tumors were of ductal origin except a single medullary sample.

Targeted primers for the amplification of ER splice variant cDNAs from normal and cancer tissues. The splice specific primers described previously (19) were used in the current study. The primers for 2Δ, 3Δ, 2-3Δ, 4Δ, 5Δ, 6Δ, and 7Δ were ER SX1/3, 5' CGCCGGCATTCTACAG 1/3 GACAT 3' (positions, exon 1, bp 669-684, and exon 3, bp 876-880), ER SX2/4, 5' AAGAGAAGTATTCAAG 2/4 GGATA 3' (positions, exon 2, bp 860-875 and exon 4, bp 993-997), ER SX1/4, 5' GCCGGCATTCTACAG 1/4 GGATAC 3' (positions, exon 1, bp 670-684 and exon 4, bp 993-998), ER SX3/5, 5' GTGGGAATGATGAAAGGTG 3/5 GCTTT 3' (positions, exon 3, bp 974-992 and exon 5, bp 1329-1333), ER AX4/6, 5' ATTTTCCCTGGTTC 6/4 CTGGCAC 3' (positions, exon 6, bp 1481-1468 and exon 4, bp 1328-1322), ER AX 5/7, 5' CAGAAATGTGTACACTC 7/5 CTGT 3' (positions, exon 7, bp 1618-1603 and exon 5, bp 1468-1465) and ER AX6/8, 5' CTCCATGCCTTTGTTA 8/6 CAGAA 3' (positions, exon 8, bp 1801 to 1786 and exon 6, bp 1601-1597) respectively. The following partner primers were used: 1) ER1A, 5' CCAGCAGCATGTCGAAGATC 3' (exon 6, bp, 1520-1501) for ER SX1/3 and ER SX1/4, 2)

ER2A, 5'GCACTTCATGCTGTACAGATGC 3' (position, exon 8, bp 1822-1801), for ER SX2/4 and ER SX3/5, 3) ER1S 5' ACGAAGTGGGAATGATGAAAG 3' (position, exon 3, bp 969-989) for ER AX4/6, and ER AX 5/7 and 4) ER2S, 5' AAGGCCTTCTTCAAGAGAAG 3' (position, exon 2, bp 849-868) for ER AX6/8. The sequence and locations of all the primers described here are based on the full length ERα cDNA sequence published by Green and others (25).

PCR. Polymerase Chain Reactions were performed in an automatic thermal cycler (MJ Research) as described previously (26, 27). Typically, a PCR was conducted using the HotStart-Taq PCR kit reagents in a 12.5 µl reaction volume containing the cDNA reverse transcribed from 125 ng of total RNA, 1 X PCR buffer, 1 X Q solution, 200 µM each of dNTPs, 2 µM each of sense and anti-sense primers and 0.6 U of Taq polymerase. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified using a sense primer, 5' AAGGCTGAGAACGGGAAGCTTGTCATCAAT 3' (position, exon 3, bp 241-270), an anti-sense primer, 5' TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3' (position, exon 7, bp 740 - 711) (28) and cDNA prepared from reverse transcription of 50 ng of total RNA. PCR conditions were initial denaturation for 1 min at 95⁰ C followed by 94⁰ C for 1 min, annealing for 1 min at the specified temperature depending on the primer pair used, extension for 2 min at 72⁰ C for 40 cycles and final extension for 10 min at 72⁰ C. The annealing temperature for 2Δ, 2-3Δ, 4Δ and 6Δ specific primers was 61⁰ C, for 3Δ and 7Δ specific primers was 55⁰ C, and for 5Δ primer was 65⁰ C. MCF-7 cDNA was used as a positive control in all the PCRs.

Detection and sequence analysis of PCR products. Aliquots (10 µl) of PCR amplified alternatively spliced ERα products were electrophoresed in either 1% agarose gels in Tris-EDTA buffer or 6% acrylamide gels in Tris-Borate EDTA buffer and detected by ethidium bromide staining. To detect the PCR products of GAPDH, 1 µl was electrophoresed in 6% acrylamide

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gels. To determine the identity of the PCR amplified ER products, they were electrophoresed in 1.2% agarose gels and purified individually using the QIAquick gel extraction kit. The purified products were cloned into pCR®2.1-TOPO vector as described previously (19) and sequenced by cycle sequencing method on an automated DNA sequencer (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

Statistical Analysis. The data were analyzed for any statistically significant differences between normal and cancer tissues. For this, the total number of exon deletion variants in a tissue was obtained by adding all the variants present in that particular tissue. The possible range of ERα variants was 0-15. The number of multiple exon deletion ERs was calculated by adding the variants that have two or more exon deletions, e.g., exons 2-3Δ, exons 2 &4 Δ, exons 7, 3-5Δ. The possible range of multiple exon deletion ERs was 0-9. Independent t-test was used to compare the mean number of total exon deletion ERs and also multiple exon deletion ERs between cancer and normal tissues. Multiple regression analysis was used to find any relationship between total number of exon deletion ERs and age, lymph node status (yes/no), and grade (I-III). All statistical analyses were performed using Statistical Analysis System (SAS). Fishers Exact Test was used to test the difference between groups using the Arcus software package for Windows (Research Solutions, Cambridge, UK). Results were considered to be significant at a probability level (*P*) of ≤ 0.05.

RESULTS

We analyzed the prevalence of ERα single-, double-, and multiple exon deletion transcripts in 35 mammoplasty-, and 38 breast cancer tissues by RT PCR using primers that were targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2-3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The seven targeted primers amplified a total of fifteen different alternatively spliced

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ERα mRNAs that have deletions in various combination of exons in both normal and cancer tissues. The frequency of expression of the fifteen alternatively spliced ERα mRNAs in normal and malignant breast tissues is described below and shown in Figures 1-7.

Exon 2Δ mRNAs are the most commonly occurring transcripts. We first employed the exon 2Δ targeted primer, ERSX1/3, and an anti-sense primer in exon 8 to amplify the transcripts that have exon 2 deletions as described previously (19). However, amplification was not efficient either in normal or cancer tissues presumably due to large size of the amplicon (960 bp) between these two primers and/or small amounts of these transcripts in the tissues. Therefore, we used an anti-sense primer in exon 6 (ER1A) in combination with ERSX1/3. The results obtained with this primer pair are summarized in Figure 1. We did not detect any products in four mammoplasty tissues (Type I). Thirty one of thirty five mammoplasty (normal) tissues and all thirty eight cancer tissues showed the presence of exon 2 deletion mRNAs. However, there are major differences between the normal and cancer tissues in the distribution of single, double and multiple exon deletion mRNAs in combination with exon 2 deletion. Among the mammoplasty samples, six showed exclusively single exon 2 deletion mRNAs (Type II), sixteen amplified exon 2Δ (660 bp) and exons 2, 4-5Δ transcripts (185 bp) (Type III) and five showed the presence of only 2, 4-5Δ (Type IV). Four normal tissues showed four products that have deletions in exon 2, exons 2 & 4 (324 bp) , exons 2 &5 (521 bp) and exons 2, 4-5 (Type V). Of the 38 cancer tissues analyzed only one showed the presence of single exon 2 deletion variant. Sixteen gave Type III, fourteen showed Type IV and seven amplified Type V patterns (Figure 1). Compared with breast tumors, a significantly higher proportion of normal breast tissues displayed Type I or Type II phenotypes ($P = 0.05$). By contrast, tumors were more likely to show Type IV phenotype ($P = 0.035$ tumors vs normal). The mRNAs with deletions in exons 2 & 7, exons 2, 5 & 7 and exons 2, 4-6 that were identified previously in breast cancer cell lines (19) could not be detected because of the position of the partner primer used here. It is possible that the exons 2 & 5Δ observed here

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is from exons 2, 5 & 7Δ transcript.

Frequency of exon 3Δ transcript expression is increased in tumor tissues. The distribution of exon 3Δ transcripts in normal and cancer tissues is summarized in Figure 2. As seen in the figure, twenty normal tissues and twelve cancer tissues did not express any products (Type I). Fifteen mammoplasty tissues and twelve cancer tissues expressed exon 3Δ (845 bp) transcripts (Type II). Fourteen cancer tissues showed the presence of both exon 3Δ and exons 3 & 7Δ (661 bp) (Type III). As seen in the figure, the frequency of single exon 3 deletion variant is increased in cancer tissues in addition to the expression of the double exon deletion variant, exons 3 & 7Δ ($P = 0.000032$).

Exons 2-3Δ mRNAs are more frequently expressed in cancer tissues. We previously employed 2-3Δ targeted sense primer, ERSX1/4, in combination with an anti-sense primer in exon 8 and amplified three transcripts of sizes, 840 bp, 660 bp and 520 bp, corresponding to exons 2-3Δ, exons 2-3 & 7Δ, and exons 2-3, 5 & 7Δ respectively in breast cancer cell lines. However, amplification of these transcripts with the above primer pair was not efficient as in the case of exon 2Δ, presumably for the same reasons. Therefore, we employed an anti-sense primer in exon 6, ER1A, together with ERSX1/4 to detect exon 2-3Δ sequences in mammoplasty and cancer tissues. The results generated with this primer pair are described in Figure 3. Only six of the thirty five mammoplasty tissues amplified the exon 2-3Δ variant (542 bp) (Type II) and one tissue showed the presence of a triple exon deletion variant, 2-3 &5Δ (403 bp) (Type IV) (Figure 3). On the other hand, thirteen cancer tissues amplified this double exon deletion variant (Type II). An additional six tumors showed the presence of exons 2-3Δ plus a triple exon deletion variant, exons 2-3, & 5Δ (Type III) ($P = 0.025$ tumor vs normal) and one tumor sample showed the presence of only the triple exon deletion variant (Type IV) (Figure 3). The 2-3 & 5Δ observed here is probably from 2-3, 5 &7Δ.

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There was no significant change in exon 4Δ transcripts between normal and cancer tissues. The expression of exon 4Δ transcripts in normal and cancer tissues is shown in Figure 4. Exon 4 deletion products were not detected in 20 mammoplasty and 29 cancer tissues (Type I). Five of thirty five mammoplasty tissues and 4 of thirty eight cancer tissues showed the presence of single exon 4 deletion variant (512 bp) (Type II). Ten normal and five cancer tissues showed the presence of an additional double exon deletion variant, exons 4 & 7Δ (328 bp) (Type III). A number of tumors which did not amplify for exon 4Δ transcripts with ERSX1/5 and ER2A primer pair gave exons 4 & 7Δ bands by ERAX6/8 and ER2S primers (as shown later in Figure 7). It appears that the anti-sense exon 7Δ splice targeted primer is more efficient in amplifying the exons 4 & 7Δ sequences than exon 4Δ targeted primer.

Exon 5 deletion ERα mRNAs are present at very low levels. We previously detected three different mRNAs that have deletions in exon 5, exons 2 & 5 and exons 2-3 & 5 (sizes, 730 bp, 540 bp and 430 bp respectively) using ERAX4/6 and a sense primer in exon 1 in breast cancer cell lines (19). However, none of the normal or tumor samples amplified any product when the above primer pair was used. We rationalized that it could be due to low levels of 5Δ transcripts. Therefore, we designed another sense primer in exon 3 (ER1S) that amplifies a shorter product and tested all the normal as well as cancer tissues. We amplified an expected 360 bp single exon 5 deletion product in nineteen normal tissues and 25 tumor tissues (Type II) (Figure 5) and found no significant difference between the two tissue sets.

Exon 6Δ ERα mRNA is the least prevalent. We detected a 500 bp exon 6Δ product in only one mammoplasty tissue and seven cancer tissues with 6Δ targeted primer, ERAX5/7, and ER1S (Figure 6). It appears that the exon 6Δ transcripts are the least prevalent.

A majority of normal as well as tumor tissues co-express exon 7Δ mRNAs along with

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transcripts that have double, triple and quadruple exon deletions in combination with exon 7 deletion. The profiles of exon 7Δ deletion variants in mammoplasty and cancer tissues amplified by ERAX6/8 and ER2S are shown in Figure 7. Ten normal and fifteen tumor tissues did not amplify any products (Type I). A large number of normal as well as cancer tissues showed the presence of four products that correspond to single, double, triple and quadruple exon deletion mRNAs, exon 7Δ, exons 4 & 7Δ, exons 7 &.3-4Δ, and exons 7 & 3-5Δ respectively (sizes 768 bp, 432 bp, 315 bp and bp 176 respectively) (Type II). However, due to the limitations associated with simultaneous amplification of several templates by the same primer pair in a PCR reaction, not all four products as shown in Type II profile were observed at all times. The less abundant double and triple exon deletion variants were interchangeably absent as shown in Type III pattern (Exon 7Δ, exons 7, 3-4Δ and exons 7, 3-5Δ) and MCF-7 cell line (Figure 7). Thus, Type III pattern was also observed for Type II samples. The presence of exons 7, 3-4Δ and exons 7, 3-5Δ in every tissue that has single exon 7 deletion suggests that after initial exon 7 deletion in a transcript, deletion of exon 3 is initiated and may proceed up to exon 4 or 5. The exons 2 & 7Δ, exons 2, 5 & 7Δ, exons 2-3 &7Δ and exons 2-3, 5 & 7Δ were not detected here because of the position of the sense partner primer in exon 2. However, the above transcripts and exons 3 &7Δ were also not detected previously in breast cancer cell lines with a sense primer in exon 1 and ERAX6/8, presumably due to competition among various templates.

Tumor tissues significantly express higher number of multiple exon deletion ERα mRNAs and total number of all variants. When we statistically analyzed the data to test whether the total number of both single and multiple exon deletion variants were greater in tumor samples compared to normal tissues, we found that the mean number of exon deletion ERs were significantly higher among tumors than that of normal tissues ($p < 0.044$). In addition, the mean number of multiple exon deletion ERs was also significantly larger than that of normal tissues ($p < 0.019$). However, mean number of exon deletion ERs was not related to age, lymph node

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status and grade of tumor.

DISCUSSION.

There has been a considerable degree of interest among researchers in alternatively spliced estrogen receptors since they were detected along with the wild type receptors in virtually every estrogen responsive tissue including the breast. This interest has increased in recent years since the detection of translated products of some of the splice variants in cancer tissues/cell lines (4, 5, 9) and variant mRNAs from cancer cell lines could be translated *in vitro* (6, 9, 10). Because of their presence in normal tissues, it was speculated that alternatively spliced variants are naturally occurring molecules, therefore, may have biological function in the estrogen induced signal transduction processes as positive/negative modulators of gene expression (12). However, several questions remain unclear about the splice variant receptors. Firstly, it is not known whether all variants are translated and processed *in vivo* into functionally active proteins and play a role in the estrogen-induced signal transduction process(es). Secondly, it is also not known exactly the types of all the alternatively spliced variants expressed in normal tissues and their modifications in cancer tissues. Thirdly, whether the alterations in the variant profiles contribute to the malignant transformation and clinical characteristics is not known. Finally, it is not known whether expression of splice variants is related to various stages of tissue development, reproductive cycles or age.

To address some of the above questions, we examined the expression of ERα splice variants in normal and malignant breast tissues at their mRNA levels by RT PCR methods using the newly developed 'Splice Targeted Primer Approach' that detects each category of splice variants as separate gene populations. As shown in Figures 1-7, at least 15 different types of alternatively spliced ERα mRNAs were identified in normal as well as cancer tissues. Although

several reports exist in the literature about splice variant ERα mRNA expression in breast cancer tissues, ours is the first comprehensive report showing the frequency of expression of fifteen different variants in normal breast tissues and their modifications in cancer tissues. It is possible that other researchers did not detect all the variants previously (14-17) because of the limitations posed by co-amplification of variant transcripts with wild type receptor. Among the fifteen variants identified, the transcripts that have deletions in combination with exon 2 were the most prevalent in both normal and cancer tissues studied suggesting that exon 2 is the first exon to be deleted in an ERα mRNA. A considerable number of both normal and cancer tissues do not seem to express the single exon 7Δ, which was thought to be a naturally occurring ER mRNA. None of the thirty five normal tissues studied showed the presence of every identified variant suggesting that they may not be required for normal cellular function. It is possible that these molecules arise due to cellular mistakes rather than natural processes.

While our results show that a majority of normal tissues express only the single exon deletion transcripts with the exception of those in combination with 2Δ and 7Δ, tumor tissues, on the other hand, showed increased frequency of mRNAs that have multiple deletions in various combinations of exons (p<0.019). In addition, malignant tissues also showed increased frequency of mRNAs that have deletions in consecutive exons such as exons 2, 4-5Δ and exons 2-3Δ, (Figures 1, and 3). In addition to the presence of multiple exon deletion variants, cancer tissues also showed increased expression of all variants. On an individual basis, the total number of exon deletion mRNAs are significantly higher in cancer tissues compared to normal tissues (p<0.044). Interestingly, all the nine ERα-negative tissues showed the presence of splice variants, although wild type receptor was not detected. Six of the nine tissues analyzed had mRNAs with deletions in consecutive exons in additions to single exon deletion mRNAs. They were exons 7, 3-4Δ, 7, 3-5Δ and 2, 4-5Δ, two of these had an additional exons 2-3Δ mRNA. Three tissues had only exons 2, 3-4Δ and 2-3Δ mRNAs. The significance of increased number of multiple exon deletion

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variants and those with deletions in consecutive exons in cancer tissues is not known. It is possible that multiple exon deletion mRNAs are likely intermediates in the progressive loss of exons which may ultimately lead to the loss of the whole receptor molecule in some breast cancer tissues. However, it is not clear whether the multiple exon deletion variants and the increased number of all the variants contribute to the genesis and progression of breast cancer. Of note, a recent study comparing ERα variants in small (<15 mm) screen detected breast cancers with that of benign breast lesions, has suggested that these variants may be a consequence rather than a cause of breast cancer progression (17). Nonetheless, increased frequency of exon deletion of ERα mRNA appears to be one of the modifications associated with malignant transformation of the breast tissue.

In vitro functional studies conducted with six individual single exon deletion variants in mammalian cells have shown that only exon 3Δ and exon 5Δ proteins translocate into the nucleus and are functionally active (12). The exon 3Δ protein binds ligands, has a positive transactivating property on AP-1 site and inhibits transactivation of wild type ERα by forming heterodimers and competing for Steroid Receptor Coactivator 1e (SRC-1e). The exon 5Δ appears to have a modest constitutive transactivating activity in the absence of any ligand and also inhibits the wild type receptor by competing for SRC-1e. These two receptors seem to completely inhibit the wild type receptor if present at equimolar concentrations. Based on these studies, it was proposed that exon 3Δ and exon 5Δ variants act as regulators of gene transcription (12). Our results showed that neither exon 3Δ nor exon 5Δ mRNAs were expressed uniformly in every normal tissue indicating that these two receptors are not required for positive/negative regulation of gene transcription by the wild type receptor in normal tissues. On the other hand, those normal tissues which express either exon 3Δ, or exon 5Δ or both ERs may have differential wild type receptor transactivating properties. *In vitro* studies also indicated that the exon- 2Δ, 4Δ, 6Δ, and 7Δ variant proteins remain in the cytoplasm and functionally inactive in ligand binding and transactivation. However,

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it is not known whether these splice variant proteins have any functional effects on the activation of wild type receptors in the cytoplasm. Since cancer tissues predominantly express multiple exon deletion variants, it remains to be established whether proteins are translated from these mRNAs and folded into functionally stable conformations, and have any functional effects on estrogen-mediated processes.

The composition of ER isoforms in every tissue seem to be unique in terms of wild type ERα, and alternatively spliced ERα. Wild type ERβ was also detected (data not shown) and it remains possible that splice variants of ERβ may also exist. This should be the focus of further studies. Since each receptor isoform appears to have a distinct functional property, the profile of various ER isoforms and their relative amounts in a tissue may ultimately dictate the degree of response to a particular ligand, in cellular proliferation and differentiation. The ER isoform heterogeneity may also have important clinical implications in the risk of developing breast cancer and in selecting preventive measures such as tamoxifen as a breast cancer prevention drug. The composition of ER isoforms may also have implications in designing SERMS (Selective Estrogen Receptor Modulators) to treat breast and other reproductive cancers and tissue specific estrogens and anti-estrogens.

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FIGURE LEGENDS

Figure 1. ERα exon 2Δ mRNA expression in normal and malignant breast tissues. The tissue cDNAs were analyzed for exon 2Δ profiles as described in materials and methods section. Total number of [1]normal and [2]cancer tissues analyzed for exon 2Δ here and other transcripts described in figures 2-7 were 35 and 38 respectively. The number of normal and cancer tissues expressing each type of profile and their identities are shown. Lane M here and in all the following figures has Gibco-BRL 100 bp ladder. Normal breast tissues seem to display type I or type II phenotypes. By contrast, tumors were more likely to show type III and IV phenotypes.

Figure 2. ERα exon 3Δ mRNA prevalence in normal and malignant breast tissues. The cDNAs from the normal and cancer tissues were amplified as described in the text. A majority of normal tissues did not express any exon 3Δ transcripts and cancer tissues showed increased expression of both single and double exon deletion transcripts.

Figure 3. Exons 2-3Δ profiles in mammoplasty and breast cancer tissues. The prevalence of exons 2-3Δ transcripts in mammoplasty and cancer tissues was analyzed as described in the experimental section. Cancer tissues showed increased frequency of the double deletion variant, exon 2-3Δ, and an additional triple exon deletion variant 2-3 & 5Δ.

Figure 4. Exon 4Δ expression in mammoplasty and breast cancer tissues. The presence of exon 4Δ transcripts in mammoplasty and breast cancer tissues was analyzed as
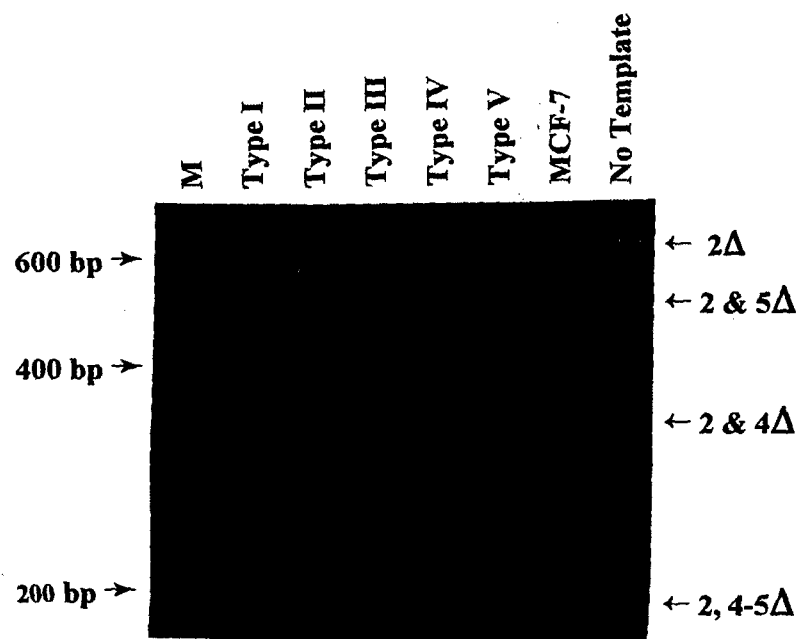
described in the text. There was no significant change in the exon 4Δ transcripts in normal and tumor tissues.

Figure 5. **Exon 5Δ transcripts in normal and cancer tissues of the breast**. The tissue cDNAs were analyzed for the presence of 5Δ mRNAs by RT PCR as described in the text. A considerable number of both normal and cancer tissues expressed this variant although at very low levels.

Figure 6. **Exon 6Δ transcripts in normal and cancer tissues of the breast**. The tissue cDNAs were analyzed for the presence of 6Δ mRNAs by RT PCR as described in the text. Exon 6Δ transcripts were the least prevalent transcripts in both normal and malignant breast tissues.

Figure 7. **ERα exon 7Δ prevalence in normal and cancer tissues of the breast**. The exon 7Δ prevalence in mammoplasty and breast cancer tissues was analyzed as described in materials and methods. The total number of normal and cancer tissues expressing the Type II or Type III is indicated. The identities of all the four products are shown.

ERα 2Δ Distribution in Breast Tissues

	M	Type I	Type II	Type III	Type IV	Type V	MCF-7	No Template
600 bp →								← 2Δ
								← 2 & 5Δ
400 bp →								
								← 2 & 4Δ
200 bp →								← 2, 4-5Δ

[1]Normal	4	6	16	5	4
[2]Cancer	0	1	16	14	7

Number Expressing the Variant

GAPDH

ERα 3Δ Distribution in Breast Tissues

	No Template	M	Type I	Type II	Type III	MCF-7	
800 bp →							← 3Δ
600 bp →							← 3 & 7Δ
400 bp →							

	Type I	Type II	Type III
[1]Normal	20	15	0
[2]Cancer	12	12	14

Number Expressing the Variant

GAPDH

ERα 2-3Δ Distribution in Breast Tissues

Type I M Type II Type III Type IV MCF-7 No Template

700 bp →
400 bp → ← 2-3Δ
          ← 2-3 & 5Δ
200 bp →
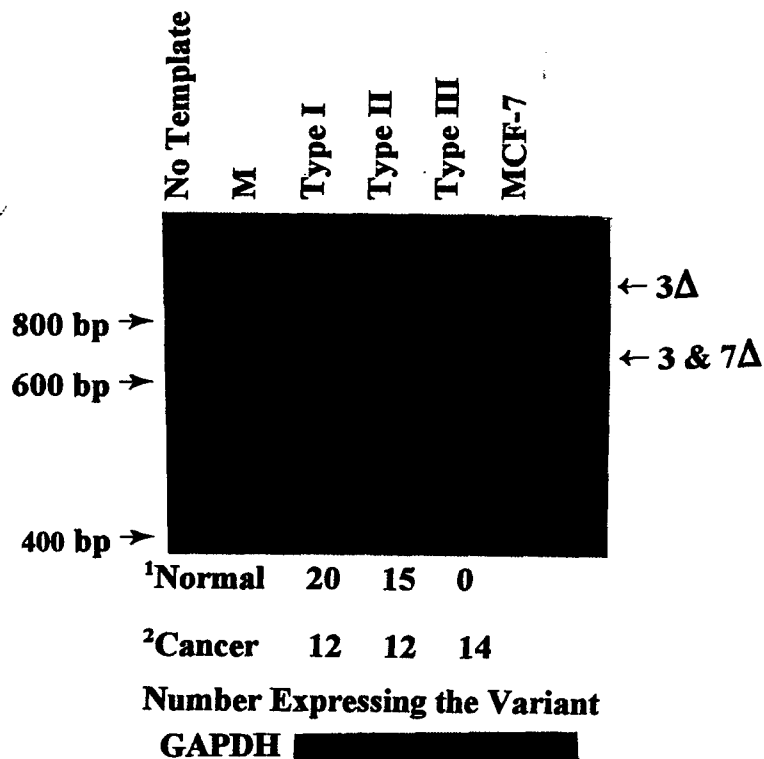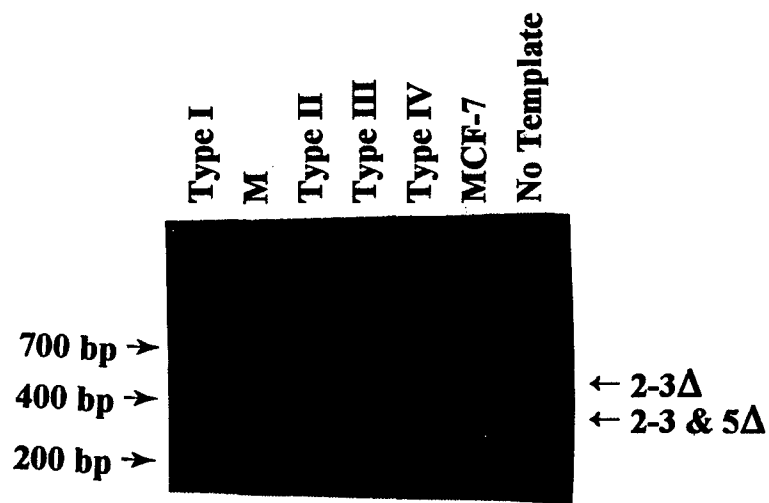
[1]Normal 28   6   0   1
[2]Cancer 18   13   6   1
Number Expressing the Variant

GAPDH

ERα 4Δ Distribution in Breast Tissues
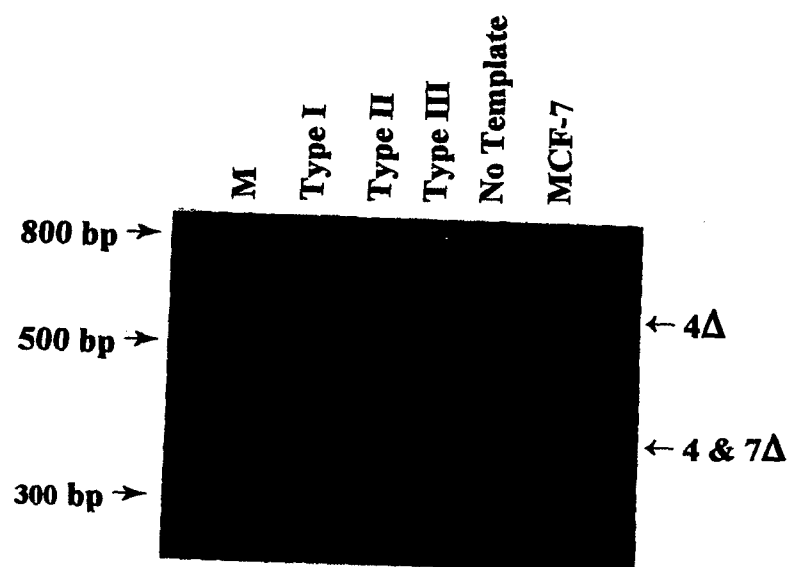
	M	Type I	Type II	Type III	No Template	MCF-7
800 bp →						
500 bp →						← 4Δ
						← 4 & 7Δ
300 bp →						
[1]Normal		20	5	10		
[2]Cancer		29	4	5		

Number Expressing the Variant
GAPDH

ERα 5Δ Distribution in Breast Tissues

No Template   MCF-7   M   Type I   Type II

600 bp →

400 bp →

← 5Δ

200 bp →

			Type I	Type II
[1]Normal			16	19
[2]Cancer			13	25

Number Expressing the Variant

GAPDH

ERα 6Δ Distribution in Breast Tissues

No Template
MCF-7
M
Type I
Type II

700 bp →

500 bp →
← 6Δ

300 bp →
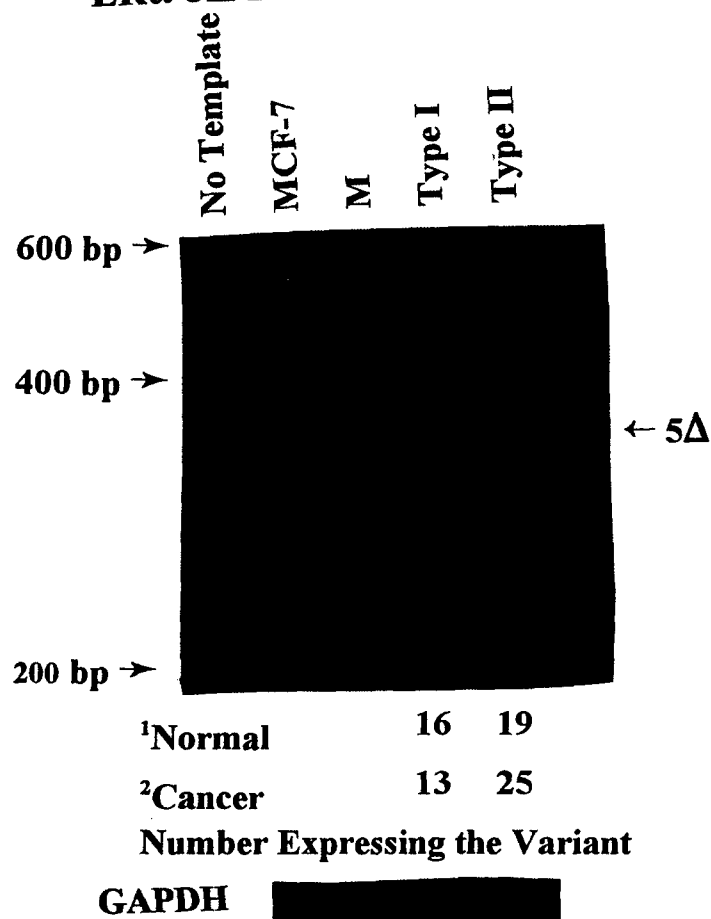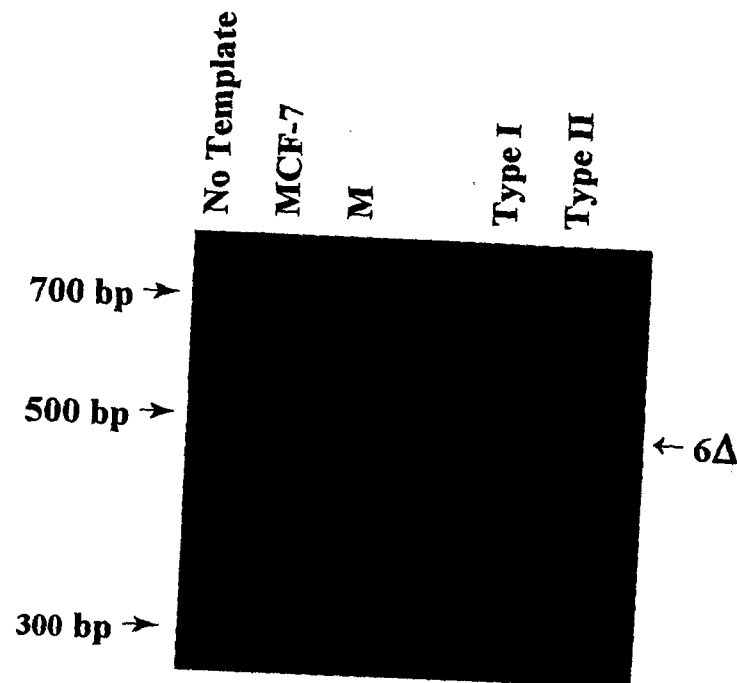
	Type I	Type II
[1]Normal	34	1
[2]Cancer	31	7

Number Expressing the Variant

GAPDH

ERα 7Δ Distribution in Breast Tissues

M Type I Type II Type III MCF-7 No Template

800 bp →

300 bp →

100 bp →

← 7Δ
← 7 & 4Δ
← 7& 3-4Δ
← 7& 3-5Δ
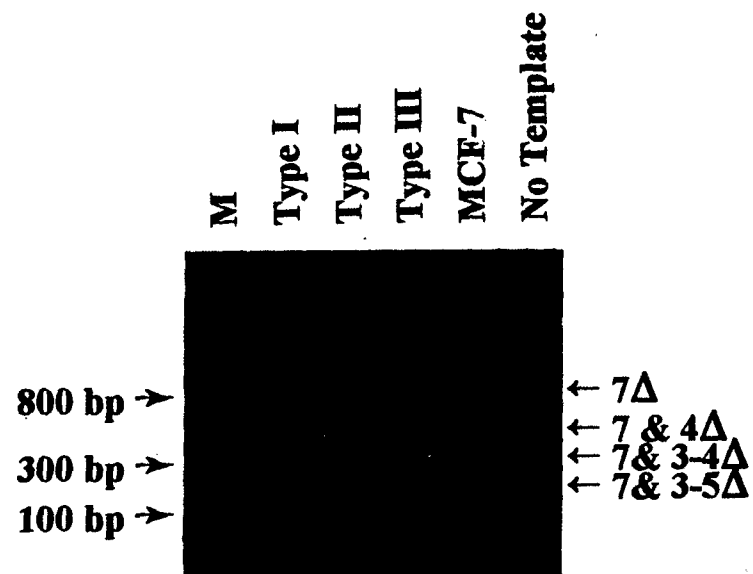
	Normal	Cancer
[1]Normal	10	25
[2]Cancer	15	23

Number Expressing the Variant

GAPDH

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   17 Oct 01

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

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FOR THE COMMANDER:

                            PHYLIS M. RINEHART
                            Deputy Chief of Staff for
                               Information Management

Enclosure